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ABSTRACT

The goals of this research were to 1) provide a basic understanding of PCB dechlorination extant in marine and estuarine environments, and 2) train graduate students in the theoretical and practical applications of environmental and anaerobic microbiology. In the course of the ONR (AASERT) funded research, the P.I. and the students have effectively identified potential PCB-dechlorinating microbes without isolation. Milestones of this research include: i) discovery of *ortho* dechlorination of PCBs, ii) development of the first defined microbial population that reproducibly *ortho*-dechlorinates PCB congeners in the absence of sediment, iii) the 16S rDNA fingerprinting of PCB dechlorinating communities by denaturing gradient gel electrophoresis, and iv) 16s rDNA-based phylogenetic characterization of individual species in *ortho-*, *para-*, and *meta*-PCB-dechlorinating cultures. In addition, two students received training in environmental microbiology over the course of this grant. They have or soon will graduate (one Masters and one Ph.D.) and have received awards based on their work. Due to these efforts the laboratory of the PI is now very close to isolating PCB-dechlorinating microorganisms. This will enable the PI and collaborators to: i) determine physiological parameters that enhance or limit PCB dechlorination; and ii) design species-specific molecular probes to screen for PCB-dechlorinating potential *in situ*.

FINAL REPORT

Grant#: N00014-96-1-1033

PRINCIPAL INVESTIGATOR: Harold D. May, Ph.D.

INSTITUTION: The Medical University of South Carolina

E-MAIL: mayh@musc.edu

GRANT TITLE: Graduate Training in Environmental and Marine Microbiology

AWARD PERIOD: 1 June 1996 - 31 May 1999

SCIENCE OBJECTIVES: The objectives were: 1) provide a basic understanding of PCB dechlorination extant in marine and estuarine environments, 2) identify bacteria that mediate anaerobic PCB transformation in order to understand the physiological pathways of the process, and 3) provide information on the physiological factors that enhance and limit the process to determine which portion of the degradative process would be potentially amenable to biotechnological enhancement.

TRAINING OBJECTIVES: Graduate students were to be trained in: 1) the theoretical and practical applications of environmental microbiology and anaerobic microbiology, 2) experimental design and troubleshooting, 3) the execution of specific chemical and molecular analyses (e.g. HPLC, GC, PCR, RFLP, DGGE), and 4) scientific writing of peer-reviewed journal articles and the presentation of research at scientific meetings. One Ph.D. student, Ms. Leah Cutter, received a stipend from this grant. Both Ms. Cutter and a Masters student, Mr. Robert S. Norman, used supply funds from this grant to pursue their science and training objectives. The AASERT award was coupled with grant N00014-96-1-116, which was awarded to H. May.

APPROACH: Ms. Cutter and Mr. Norman combined traditional microbiology techniques for the enrichment and isolation of PCB-dechlorinating anaerobes with alternative molecular approaches to identify these microorganisms and to monitor the physiological interactions and population dynamics of consortia that catalyze the transformation. The first approach included the application of single congeners and selective inhibitors to enrich for dechlorinating microbes followed by analysis of PCBs and anaerobic metabolites. The second approach included the amplification of rRNA genes by the polymerase chain reaction plus analysis by denaturing gradient gel electrophoresis.

ACCOMPLISHMENTS: In the course of the three years of ONR (AASERT) funded research, the P.I. and the students have effectively identified potential PCB-dechlorinating microbes without isolation. Milestones of this research include: i) discovery of *ortho* dechlorination of single PCB congeners, ii) development of the first defined microbial population that reproducibly *ortho*-dechlorinates PCB congeners in the absence of sediment, iii) the 16S rDNA fingerprinting of PCB dechlorinating communities by denaturing gradient gel electrophoresis, and iv) 16s rDNA-based phylogenetic characterization of individual species in *ortho-*, *para-*, and *meta*-PCB-dechlorinating cultures. Furthermore, the metabolic requirements of the *ortho*-dechlorinating culture were determined.

CONCLUSIONS: The two students received a high degree of training in environmental microbiology over the course of this grant. They have or soon will graduate and have received awards based on their work (see below). The research has demonstrated that a combination of enrichment technique and molecular monitoring would result in highly defined and selective PCB-dechlorinating microbial populations in a defined minimal medium. The approach has resulted in the identification of organisms associated with these activities and has proven the worth of rapid screening of such cultures by denaturing gradient gel electrophoresis.

SIGNIFICANCE: Due to these efforts the laboratory of the PI is now very close to isolating PCB-dechlorinating microorganisms. This will enable the PI and collaborators to: i) determine physiological parameters that enhance or limit the dechlorination process; and ii) design species-specific molecular probes to screen for PCB-dechlorinating potential in cultures and *in situ*.

PATENT INFORMATION: Bacterial Dechlorination of Polychlorinated Biphenyls (PCBs). Mary Berkaw, Leah A. Cutter, Margaret Elberson, Tracey Holoman, Harold D. May, Tracey Holoman, Kevin R. Sowers. Currently being reviewed by tech transfer departments by the University of Maryland (Sowers is collaborator with H. May on other ONR grants).

AWARD INFORMATION: Following the training received during the support of this grant and under the mentorship of the PI (H. May) the following students have earned their graduate degrees, received awards or are on track to graduate in 2000.

Robert S. Norman, Masters in Environmental Sciences, graduated in May of 1999 from the Medical University of South Carolina/University of Charleston Environmental Studies Program. He was honored as the Most Outstanding Student within the program for 1999. His thesis was entitled: "Molecular Determination of the Microbial Community Structure Associated with the Dechlorination of 2,3,4,5-tetrachlorobiphenyl".

Leah Cutter, Ph.D. candidate, has passed all of her qualifying and preliminary exams. She has published two manuscripts from her dissertation work thus far and is planning 2+ more. In addition, she won First Prize in Oral Presentation Session (third year graduate students) at MUSC Student Research Day (1998). She is on track to defend her dissertation and graduate with a Ph.D. by the end of 2000.

PUBLICATIONS AND ABSTRACTS (for total period of grant):

1. Berkaw, M., L. Cutter, K.R. Sowers, and H.D. May. 1996. Site-dependent *ortho-, meta-, and para* -dechlorination of PCBs by anaerobic estuarine and marine sediments enrichments. Abstr. 5th European Marine Microbiology Symposium, Bergen, Norway.

2. Berkaw, M., L. Cutter, K.R. Sowers, and H.D. May. 1996. Anaerobic *ortho*-Polychlorinated Biphenyl Dechlorination by Estuarine and Marine Sediments. Abstr. 96th Ann. Mtg. Amer. Soc. Microbiol., p. 71, Q-189.

3. Cutter, L., M.A. Elberson, K.R. Sowers, and H.D. May. 1996. Selective Enrichment for Anaerobic PCB Dechlorination. South Carolina Branch

Meeting of the American Society for Microbiology.

4. Cutter, L., K.R. Sowers and H.D. May. 1997. Selective Enrichment for PCB-Dechlorinating Anaerobes from Estuarine Sediments. Abstr. 97th Ann. Mtg. Amer. Soc. Microbiol., p. 479, Q-142.

5. Holoman, T., M.A. Elberson, L.A. Cutter, H.D. May and K.R. Sowers. 1998. Characterization of Selective ortho PCB-Dechlorinating Enrichment Cultures by Comparative Analysis of 16S rDNA. Abstr. 98th Ann. Mtg. Amer. Soc. Microbiol., p. 473, Q-317.

6. Holoman, T., L. Cutter, H.D. May and K.R. Sowers. 1998. Molecular characterization of Baltimore Harbor enrichment cultures capable of *ortho*-dechlorination of 2,3,5,6-tetrachlorobiphenyl. Environmental Risk Reduction via Biotechnology, Abstr. Ann. Amer. Inst. Chem. Engineers Mtg., Miami Beach, FL

7. Holoman, T.R.P., M.A. Elberson, L. Cutter, H.D. May and K.R. Sowers. 1998. Characterization of a defined 2,3,5,6-tetrachlorobiphenyl *ortho*-dechlorinating microbial community by comparative sequence analysis of genes coding for 16S rDNA. Appl. Environ. Microbiol. *64:* 3359-3367.

8. Cutter, L., K.R. Sowers and H.D. May. 1998. *Ortho*-dechlorination of 2,3,5,6,-chlorinated biphenyl by estuarine microbial populations in sediment-free enrichment cultures. Appl. Environ. Microbiol. *64:* 2966-2969.

9. Norman R.S., S. Schreier, J. Watts, K.R. Sowers, and H.D. May. 1999. Molecular assessment of the effect of PCBs on the microbial community structure within an enrichment culture. American Society for Microbiology Conference on Microbial Diversity.

Poster no. 66

Differential RFLP patterns of PCR-amplified 16S rDNA from anaerobic PCB-dechlorinating estuarine and marine sediment enrichments.

Elberson, M.A.[1], May, D.H.[2], and Sowers, K.R.[1]

University of Maryland Biotechnology Institute, Baltimore, MD[1] and Medical University of South Carolina, Charleston, SC[2].

Para-, meta- and *ortho*-dechlorination activities of individual polychlorinated biphenyl congeners (PCBs) by sediments from Baltimore Harbor have been maintained throughout sequential transfers of enrichment cultures. However, microorganisms responsible for dechlorination have not yet been identified by standard isolation techniques. In order to identify PCB - dechlorinating microorganisms, a procedure was developed for the isolation and PCR amplification of DNA encoding 16S rRNA from marine and estuarine enrichments containing sediments with high concentrations of humic acids. Enrichment slurries were lysed with glass beads in phosphate burrer containing Denhardt's solution and the DNA was extracted with phenol-chloroform. Humic acids associated with DNA were removed with insoluble polyvinylpyrrolidone, extracted from a low-melt agarose gel containing soluble polyvinylpyrrolidone, then amplified by PCR using universal oligonucleotide primers. DNAs encoding 16S rRNA from Archaea (*Methanosarcina thermophila)*, Bacteria (*Escherichia coli)*, and Eucarya (*Morone saxatilis)* are recovered and amplified from as few as 10^2 cells in sediment slurry. Differential RFLP patterns from PCR generated 16S rDNA are shown for enrichments that *para-, meta-* and *ortho*-dechlorinate 2,3,4,5-PCB, as well as cultures that exhibit *para-* or *ortho*-dechlorination of 2,3,4,5-PCB and 2,3,5,6-CB, respectively. Initial analyses of the gene sequences from representative RFLP patterns indicate that this approach is effective for discrimination of mixed rDNA populations in PCB-dechlorinating enrichments that are up to 98% homologous.

Fifth European Marine Microbiology Symposium
Bergen, Norway, August 11-15, 1996

Session 118.
Biodegradation of Polychlorinated Biphenyls
Tuesday, 10:30 a.m.

Q-186 Functions of Extracellular Polysaccharides of *Rhodococcus rhodochrous*. NORIYUKI IWABUCHI,† MICHIO SUNAIRI,† HISAO MORISAKI‡, and MUTSUYASU NAKAJIMA.†* †Nihon Univ., Fujisawa, Japan; ‡Ritsumeikan Univ., Kusatsu, Japan.

Rhodococcus is a versatile genus of nocardioform actinomycetes, which plays an important role for biodegradation of xenomaterials, *e.g.*, PCB. It is essential to understand its behavior in environments for the application to bioremediation. We report nature of the bacterial cell surface, *e.g.*, electrokinetic potential or hydrophobicity, which is an important determinant in the bacterial behavior.

Four colony-morphological mutants of *R. rhodochrous* (S-1, and S-2, mucoidal; R-1, and R-2, rough) produced 6.8, 14.5, 1.4, and 1.9 (mg dry EPS / g fresh cells), respectively. Their electrophoretic mobilities were almost the same negative values (-3×10^{-8} m^2/Vs) between pH 4 and 9.

Cell surface hydrophobicity was determined by five different methods, *i.e.*, MATH, contact angle, SAT, HIC and DOS. The order of hydrophobicity was determined as R-2 » R-1 » S-1 > S-2. LBM method devised for measuring cell surface hydrophobicity of mucoidal strains revealed that S-2 has hydrophobic surface covered with hydrophilic EPS, indicating that the EPS function as hydrophilin.

Next, the effect of hydrophilic EPS on adhesion of the bacterium to particles in environments was analyzed by model experiments using glass, quartz and teflon. Rough strains well adhered to the materials, whereas mucoidal strains little adhered to these materials. Sedimentation tests showed that the cells of rough strains settled within several hours, whereas mucoidal strains scarcely settled.

Mucoidal mutants appeared from rough strains, R-1 and R-2, at frequencies of 2.4×10^{-6} and 1.5×10^{-6}, respectively.

In conclusion, hydrophobic cells have the advantage for adhesion, in contrast, hydrophilic cells can be conveyed with movement of water.

Q-187 Integrating Surfactant Enhanced PCB Solubilization and Biodegradation in a Soil Remediation Process
A. C. LAYTON,* J. P. EASTER, C. A. LAJOIE, M. MUCINNI, & G. S. SAYLER. University of Tennessee, Knoxville, TN 37932.

A two phase remediation process has been developed for polychlorinated biphenyl (PCB) contaminated soils at electric utility substations. In the first phase, 80-90% of the weathered Aroclor 1248 is desorbed from the soil in situ by a two day recirculating surfactant wash (1%wt/vol). In phase two, the surfactant/PCB solution is collected in a bioreactor and amended with nutrients and the field application vectors (FAVs) *Pseudomonas putida* IPL5::TnPCB and *Alcaligenes eutrophus* B30P4::TnPCB. These strains use the surfactant as a growth substrate and contain the entire PCB degradative operon inserted on a transposon. After 1 week, >90% of the surfactant and >30% of the PCBs are degraded. The residual desolubilized PCBs are deposited on a soild carrier and removed from the bioreactor effluent (>99%). The concentrated residual PCB congeners may be partially dechlorinated by physiochemical or biological processes and recycled to the bioreactor. Toxicity testing, using *Tetrahymena* and Microtox systems, is being performed on soils and process solutions. A proposed field trial will be performed at an electric power substation pending EPA approval.

Q-188 *In Situ* Biodegradation of PCB-Contaminated Surface Soils for Reduction of Leachable PCBs.
M. J. R. SHANNON*, R. K. ROTHMEL, AND R. UNTERMAN. ENVIROGEN, INC. Lawrenceville, NJ 08648.

A two-year field demonstration of aerobic, *in situ* PCB bioremediation was completed. Two plots, each containing 3700 Kg of surface soil, were created within a greenhouse. The experimental (bioaugmentation) plot was dosed with biphenyl and PCB-degrading bacteria that exhibit complementary congener specificity (Type II and Type IV dioxygenase activities). The control plot received no cells and received a limited amount of biphenyl during the later part of the final year (biostimulation control).

The initial average PCB concentration of 39 mg/Kg was reduced by 44% to 22 mg/Kg in the experimental plot. During the 1994 season, 20% PCB degradation was achieved, most of which occurred during the first 4 weeks of treatment. Parallel laboratory experiments demonstrated that the limited PCB biodegradaqtion in 1994 was likely due to an insufficient amount of biphenyl. Additional biphenyl added in 1995 resulted in a further reduction in PCB concentration to 22 mg/kg.

Degradation during 1994 was limited to the lower chlorinated congeners (di- tri- and tetrachlorinated PCBs), and as the biological activity progressed during 1995 more extensive degradation of tetra- and pentachlorinated congeners occurred. Overall, 89% of the di-, 84% of the tri-, 51% of the tetra- and 28% of the pentachlorinated congeners were degraded by the end of the demonstration. The data show that biodegradation resulted in the destruction of soluble, bioavailable congeners, and suggest that biodegradation will result in PCB stabilization and reduced risk of PCB migration and exposure.

Q-189 Anaerobic *ortho* PCB Dechlorination by Estuarine and Marine Sediments.
BERKAW[1], M., L. CUTTER[1], K. R. SOWERS[2], AND H. D. MAY[1]*. The Medical University of South Carolina, Charleston, SC[1], and the University of Maryland Biotechnology Institute, Baltimore, MD[2].

Estuarine sediments from Baltimore Harbor *ortho*-dechlorinate a number of PCB congeners under anaerobic conditions. *Ortho* dechlorination of 2,3,4,5-CB occurs with these sediments in marine, estuarine, and freshwater media. The effects of various media on the acclimation time and on the type of dechlorination that develops (*meta*, *ortho*, or *para*) are presented. Dechlorination (*meta*, *ortho*, and *para*) most rapidly develops (<1 month) in an estuarine medium lacking sulfate. Dechlorination is delayed in marine medium or by the addition of sulfate. Use of reduced anaerobic mineral medium (RAMM), a freshwater medium, delays the onset of *ortho* dechlorination for more than a month and heavily favors *para* dechlorination. *Ortho*-dechlorinating cultures have been maintained in the absence of sediment. After 3 serial transfers (the first containing supernatant from an active sediment) several transfer cultures *ortho* dechlorinated 2,3,5-CB after the *para* dechlorination of 2,3,4,5-CB. These cultures and their requirement for, or independence from, sediment are discussed. *Ortho* dechlorination has also been observed with sediments from other locations. Sediments from five sites in Charleston Harbor, one site in the Chesapeake Bay near the mouth of the Potomac River, and one site in the Hudson River (H7) were examined for *ortho* dechlorination in marine, estuarine, and freshwater media. *Ortho* dechlorination of 2,3,5-CB or 2,3,5,6-CB was observed with 3 of the 5 Charleston Harbor sediments, however none of these developed activity as quickly as Baltimore Harbor sediments do and the type of dechlorination varies with the site and environmental conditions. The dechlorination activities expressed by sediments from all these sites are presented.

Q-190

Identification of Plants Having Potential Rhizosphere Effects on Polychlorinated Biphenyl Biodegradation.
ERIC S. GILBERT* and DAVID E. CROWLEY. Univ. of Calif., Riverside, CA 92521.

The rhizosphere microenvironment has been reported to enhance the biodegradation of xenobiotic chemicals. The potential for a rhizosphere effect on polychlorinated biphenyl (PCB) biodegradation has not been fully evaluated. As part of a study of rhizosphere influence on PCB biodegradation, a screening assay was developed to identify plants which might induce bacterially-mediated PCB degradation.

Arthrobacter sp. strain B1B, a Gram-positive bacterium known to cometabolize Aroclor 1254, was grown on selected plant extracts. Washed cell suspensions of strain B1B were prepared; 4,4'-dichlorobiphenyl subsequently was added and the rate of formation of the phenylhexadienoate ring-fission product, an indicator of PCB oxidation, was monitored spectrophotometrically. Rates of product formation after growth on plant substrates were compared to rates after growth on biphenyl, the non-chlorinated PCB analog, and on various nutrient media.

Root extracts of common plants such as rye grass (*Lolium perenne*) and green bean (*Phaseolus vulgaris*) did not stimulate ring-fission product formation, nor did compost extracts. However, a representative aromatic plant, *Mentha sp.*, proved to be an effective inducer of ring-fission product formation. 4-chlorobenzoate was identified by HPLC as a metabolite, indicating hydrolysis of the ring-fission product also occurred. These results suggest that certain plants may produce metabolites which, if present in the rhizosphere, may promote PCB cometabolism.

Q-139. Aerobic Degradation of Polychlorinated Biphenyls by Boreal Freshwater Sediment Cultures

S. HURME,* AND J. A. PUHAKKA. Tampere Univ. of Technology, Tampere, Finland

We studied the capability of indigenous freshwater sediment microorganisms to degrade polychlorinated biphenyls (PCBs) under aerobic conditions. Sediment samples were collected from a boreal lake (Lake Kernaalanjarvi, Finland) which has been exposed to a minor PCB load for several decades. Typical PCB concentrations in the sediment are around 1 mg/kg d.w, and the maximum concentrations do not exceed 5 mg PCBs/kg d.w. Aerobic biphenyl degraders, enriched from the surface sediment samples, were tested for their ability to degrade Aroclor 1242. Initial PCB concentration in batch vial experiments ranged from 25 to 200 mg/L. Aroclor 1242 did not serve as a growth substrate for the enrichment cultures, but was cometabolized in the presence of biphenyl. Selected mono-, di-, and trichlorobiphenyls were degraded resulting in the total degradation of approximately 20% over a period of one month. Congeners with a substitution pattern of either 2,2'-, 2,6-, or 4,4'-, and those containing more than three chlorine substituents resisted degradation. Biphenyl concentration affected degradation considerably. Trichlorobiphenyls present in Aroclor 1242 were resistant in the absence of biphenyl. Furthermore, the degradation typically ceased after a few days of incubation although biodegradable congeners were still present. This was explained by the depletion of biphenyl. The extent of total PCB degradation was modest. More importantly, we demonstrated that indigenous boreal freshwater sediment microorganisms, exposed to low-level PCB contamination, have the potential to degrade high concentrations of selected lower chlorinated PCB congeners in Aroclor 1242.

Q-140.

Detection of *In situ* Anaerobic PCB Dechlorinators in a Contaminated Microbial Consortium.

Robert J. Davenport, James M. Champine*, and S.K. Dutta

Department of Biology, Howard Univ., Washington, D.C. 20059, and Michigan Center for Microbial Ecology, East Lansing, MI.

A method for assessing the presence of PCB dechlorinating organisms in contaminated sediments is essential to understanding intrinsic remediation. A consortium from Lake Medinah, NY was used to establish laboratory microcosms having distinct *meta* and *para* dechlorination activities with 2,3,4-trichlorobiphenyl. DNA from the anaerobic heterotrophic bacteria, chiefly clostridia, from each community was extracted and analyzed by Amplified Ribosomal DNA Restriction Analysis. Oligonucleotide probes based on 16S rRNA genes were designed for the most abundant Operational Taxonomic Unit (OTU A and B) in each microcosm. To confirm the presence of OTU-A and OTU-B in sediment samples, the probes were used in dot blot and Southern hybridization studies. Eubacterial and Archaeal primers were used to amplify 16S rDNA from the same sediments. Interestingly, there was PCR product with the Archaea primers, indicating that Archaea, as well as members of the Genus *Clostridium* are present. Results indicate that the most predominant member of the consortium was detectable and could be used as an indicator for natural dechlorination in other sediments.

Q-141.

Anaerobic Dechlorination of Polychlorinated Biphenyls: Dynamics of Dechlorinating Microorganisms and their Interactions with Methanogens and Sulfate Reducers

DONG-KYUN KIM,[1]* AND G-YULL RHEE[1,2]

School of Public Health, Univ. at Albany, SUNY[1], and Wadsworth Ctr., NYS Dept. of Health[2], Albany, NY 12201-0509

Dynamics of PCB-dechlorinating microorganisms were determined along with sulfate reducers and methanogens using the most-probable-number technique. The time course of PCB dechlorination mirrored the growth of dechlorinators; dechlorination ensued when the dechlorinating population increased by two orders of magnitude from 2.45×10^6 to 10^8 cells g^{-1} sediment between 2 to 6 weeks. During this period, PCB-dechlorinating microorganisms dechlorinated Aroclor 1248 at a rate of 39.29×10^{-9} mole Cl g^{-1} day^{-1}, and growth yield was 41.60×10^{12} cells $mole^{-1}$ Cl dechlorinated. Once dechlorination reached a plateau after 6 weeks, the number of dechlorinators began to decline. On the other hand, dechlorinators inoculated into PCB-free sediments decreased slightly from their initial level, suggesting that PCBs are required for their selective enrichment. Sulfate reducers and methanogens increased in both PCB-free and PCB-amended sediments showing little difference between them. The potential role of methanogens and sulfate reducers on PCB dechlorination was investigated using specific inhibitors, bromoethanesulfonate (BES) and molybdate. Addition of molybdate had no effect on Aroclor 1248 dechlorination, indicating that sulfate reducers might not be directly involved in or inhibit the dechlorination process. In BES-amended sediments, *meta*-rich congeners such as 2,5,2',5'-, 2,4,2',4'-, and 2,5,2'-chlorobiphenyls were not dechlorinated; this suggests the selection of different dechlorinating populations. Interestingly, addition of molybdate and BES completely inhibited Aroclor 1248 dechlorination.

Q-142. Selective Enrichment for PCB-Dechlorinating Anaerobes from Estuarine Sediments

L. A. CUTTER,[1]* K. R. SOWERS,[2] and H. D. MAY.[1] Med. Univ. South Carolina, Charleston,[1] Univ. Maryland Biotech. Inst., Baltimore.[2]

Bacterial enrichments developed from Baltimore Harbor sediments reductively dechlorinate polychlorinated biphenyls (PCBs) when incubated under anaerobic conditions. Initial enrichments produced various ortho, meta and para products from 2,3,4,5-chlorobiphenyl (CB) and 2,3,5,6-CB when maintained in estuarine or marine media. Successive transfer of these enrichments has resulted in selection of specific products. For example, initial enrichments with 2,3,5,6-CB expressed both meta and ortho dechlorination pathways but after sequential transfer on 2,3,5,6-CB only the ortho pathway remained. Initial enrichment with 2,3,4,5-CB resulted in para- and meta-dechlorination to 2,3,5-CB and 2,4,5-CB followed by ortho-, meta- and para-dechlorination to di- and monochlorobiphenyls. Successive transfer with 2,3,4,5-CB has lead to enrichments that only produce 2,3,5-CB, 3,5-CB and 2,5-CB with 3,5-CB being the main product. The specific activities observed in transfers on 2,3,5,6-CB and 2,3,4,5-CB were maintained regardless of the amount of sediment added to the medium. Continued transfer of all enrichment lines in the absence and presence of sediments is under examination. The effects of various carbon (energy) sources and inhibitors on dechlorination and enrichment/isolation will also be discussed.

Q-143.

Reductive Dechlorination of Coplanar PCB Congeners in the Anoxic Estuarine Sediment Slurries.

C.-E. KUO[1], S.-M. LIU[1]*, and C. LIU[2].

[1]Natl. Taiwan Ocean Univ., [2]Natl Inst. of Environ. Analys., Environ. Protect. Adm., Taipei, Taiwan

Of 209 PCB congeners, 20 congeners with chlorine atom at both *para* and *meta* positions but lack complete substitution in the *ortho* position show a coplanar configuration. It had been demonstrated that these coplanar congeners are more toxic and less biodegradable than nonplanar PCB congeners. Concern over their toxicity and bioaccumulation potential have emphasized the need to clean up these coplanar PCBs.

In this study, biodegradability of 4 coplanar congeners: 3,3',4,4'-tetrachlorobiphenyl; 3,4,4',5-tetrachlorobiphenyl; 3,3',4,4',5-pentachlorobiphenyl; 3,3',4,4',5,5'-hexachlorobiphenyl were investigated by amending 10 ppm of each compound into the anoxic sediment slurries collected from the estuary of Tansui River and Er-Jen River. During 13 month incubation, the parent compounds and the intermediate products were determined with gas chromatography (GC) and GC/MS.

Except for 3,3',4,4',5,5'-hexachlorobiphenyl, all other tested coplanar congeners were dechlorinated in 10 month after a lag period of 61 days in the sediment slurries collected from Er-Jen River. However, both 3,3',4,4',5,5'-hexachlorobiphenyl and 3,3',4'4'-tetrachlorobiphenyl were persistent in the sediment slurries collected from Tansui River. Dechlorination of the other 2 congeners were much slower in the sediment slurries collected from Tansui River than those from Er-Jen River. Examination of the chromatograms over the time course of the incubation indicates that dechlorination of these congeners were initiated from *para* chlorine removal. One to three chlorines were removed from these congeners during 13 month incubation.

Q-144. Evidence of degradation and mineralization of biphenyl by anaerobic microbial consortium.

M. R. NATARAJAN*, W. Wu, R. Sanford, H. WANG and M. K. JAIN. MBI International, Lansing, Mich.

In the past, degradation of biphenyl by aerobic microorganisms has been known, but information on its anaerobic degradation has been limited. We have previously developed an anaerobic microbial consortium in granular form that was shown to dechlorinate polychlorinated biphenyls (PCBs) into biphenyl. In this study, we demonstrate degradation and mineralization of biphenyl to CO_2 and CH_4 by these dechlorinating granules under methanogenic conditions. Biphenyl was degraded to p-cresol which was further mineralized to CO_2 and CH_4. These results were obtained with labeled ^{14}C-biphenyl as well as unlabeled biphenyl and p-cresol. Production of ^{14}C-CO_2 and ^{14}C-CH_4 was found to increase during a time course study. The ratio of ^{14}C-CO_2 and ^{14}C-CH_4 in the headspace was about 1:2 after 16 weeks of incubation. The tentative anaerobic biodegradative pathway of biphenyl is proposed as: biphenyl p-cresol CO_2 + CH_4. Our results indicate existence of novel biodegradative pathways in natural anaerobic microbial community that has broad implications in the field of microbial ecology and detoxification and elimination of toxic pollutants.

Q-145. Reductive dechlorination of an ortho-substituted PCB congener by Chesapeake bay sediments acclimated to para- and meta-chlorinated congeners

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On the spectrum of xenobiotic pollutants from easiest to most difficult, polychlorinated biphenyls (PCBs) are among the most challenging for bioremediation. Often dechlorination of meta- and para- chlorine moieties proceed at a faster rate than ortho-chlorine. Last year, we reported meta- and para-dechlorination of PCBs by sediments from the Chesapeake bay and recently, Berkaw et al., have reported reductive ortho-dechlorination of PCBs by estuarine sediments from the Baltimore Harbor. In order to further characterize our sediments, we examined the ability of our microbial consortia that had been acclimated to four concentrations of a meta- and para-substituted PCB congener to reductively dechlorinate an ortho-saturated PCB congener. Anaero-

processes governing MTBE biodegradation.Controlled studies using batch incubations demonstrated that MTBE removal by the GAC was a combination of physical sorption and biological degradation. Maximum MTBE removal rates are estimated to be on the order of 4,000 mg MTBE/g GAC/day with an apparent half-saturation constant of approximately 7,000 mg MTBE/L (in the presence of GAC). Removal of MTBE by the GAC appears to be pH sensitive.Forty-nine bacterial strains were isolated from the GAC by enrichment on MTBE and plating on both selective and non-selective media. These isolated strains were grouped into nine colony phenotypes. At least two phenotype groups had representative strains that oxidized MTBE. Preliminary analysis suggests that the true half-saturation constant for the pure cultures is several orders of magnitude lower than that observed in the reactor and that the maximum specific MTBE oxidation rates are low. The significance of theses results to the biological treatment of MTBE will be discussed.

Q-315. Acrylamide Degradation by a *Pseudamonas aeruginosa* Strain

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Increasing use and indiscriminate discharge of acrylamide and other related amides is becoming a serious type of contamnants in soil and water. Higher concentrations of these amides do not degrade rapidly. The purpose of this study was to screen and isolate bacterial strains capable of degrading acrylamide efficiently. We have isolated a strain of *Pseudomonas aeruginosa* from the effluent of an explosive factory which showed excellent growth with as high as 63 mM acrylamide. Complete inhibition of growth was observed at 90 mM. Our results show that acrylamide is used as the sole sources of carbon and nitrogen for the growth of *P. aeruginosa*. Employing GLC technique, the primary product of acrylamide degradation has been identified as acrylic acid. Another metabolite in the culture filtrate was determined to be ammonia. Formation of acrylic acid and ammonia by *P. aeruginosa* revealed close correlation with the disappearance of acrylamide from the medium. Enzyme responsible for acrylamide degradation has been identified as amidase which was inducible in nature. *P. aeruginosa* appears to be a potent degrader of acrylamide and may be employed in bioremediation.

Q-316. Construction of Environmental DNA Libraries and Screening for Anaerobic Utilization of 4-Hydroxybutyrate by Recombinant *Escherichia coli* Strains

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The genetic diversity of the microorganisms in an environment offers interesting opportunities to encounter new or improved genes and gene products for biotechnological purposes. In order to exploit the genetic diversity DNA libraries of several environments were constructed. DNA was extracted from various soil samples by lysis with a salt extraction buffer and extended heating in the presence of SDS. The final purification was performed with the Wizard® Plus Minipreps DNA Purification System. The purified DNA was partially digested with *Bam*HI or *Sau*3AI, ligated in pBluescript SK and transformed into *Escherichia coli*.

The resulting recombinant *E. coli* strains were screened on tetrazolium indicator plates for the utilization of 4-hydroxybutyrate (4-HB); six out of approximately 270,000 clones were positive. These clones showed a slower growth rate on 4-HB than *E. coli* JM109/pCK1, which harbors the gene encoding 4-HB dehydrogenase from *Clostridium kluyveri*. Enzymatic analysis revealed 3-HB and 4-HB dehydrogenase activity in crude extracts of the recombinant *E. coli* strains. The inserts of the plasmids isolated from these strains were sequenced. The deduced gene products exhibited no significant similarity to any other known protein.

Q-317. Characterization of Selective ortho PCB-Dechlorinating Enrichment Cultures by Comparative Sequence Analysis of 16S rDNA

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Enrichment cultures that selectively ortho-dechlorinate 2,3,5,6-tetrachlorobiphenyl were analyzed by comparative sequence analysis of 16S rDNA genes amplified from total community DNAs in order to identify potential PCB-dechlorinating anaerobes. Population profiles are presented from enrichments that ortho dechlorinate 2,3,5,6-CB in the presence or absence of sediment. Dechlorination in the presence of fatty acids or lactate showed that different carbon sources select for different populations. Population profiles from enrichments exposed to specific inhibitors (bromoethanesulfonic acid, vancomycin, and molybdate) demonstrated that highly enriched PCB-dechlorinating microbes could be obtained. In addition, molecular monitoring showed that some highly enriched species found in dechlorinating cultures were absent in inactive cultures and in enrichment cultures developed without PCBs. By combining selective enrichment with molecular monitoring (SEMM technology), defined ortho-dechlorinating consortia have been established and maintained through sequential transfers.

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Q-318. Functional Analysis of the *Pseudomonas syringae rulAB* Determinant in Tolerance to Ultraviolet B (280 to 320 nm) Radiation and Distribution of *rulAB* Among *P. syringae* Pathovars

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The bacterial plant pathogen *Pseudomonas syringae* is adapted to growth and survival on leaves in the phyllosphere, a habitat which is normally exposed to high doses of natural UV radiation. We recently determined that the indigenous plasmids pPSR1 and pPSR5 from *P. syringae* pv. syringae contained a homolog of the *umuDC* mutagenic repair operon termed *rulAB* which functioned in tolerance to UVC (254 nm) radiation (Gene 177:77-81). In this study, we analyzed the role of *rulAB* in conferring tolerance to environmentally-relevant levels of UVB radiation both in vitro and in the phyllosphere. We also examined the distribution and UVB sensitivity of a worldwide collection of *P. syringae* pathovars. We examined the differences in survival of *P. syringae* pv. syringae FF5 containing the *rulAB* determinant cloned in pGWS157 and FF5 containing the vector control. Measured doses of UVB radiation were delivered either to cells previously grown in LB broth and resuspended in 0.85% NaCl or to populations established from one to five days in the bean phyllosphere. Our results indicated that the survival of FF5(pGWS157) was approximately ten to twenty-fold greater than FF5(vector) following irradiation of cell suspensions with a range of UVB doses (750 to 1,150 J m^{-2}). A difference in percent survival of five to ten-fold was observed in the comparison of FF5(pGWS157) and FF5(vector) following the irradiation of bean phyllosphere populations with a UVB dose of 850 J m^{-2}. This smaller difference was attributed to the ability of a portion of the total FF5 population on bean to access sites within bean leaves protected from the UVB dose. Analysis of the UVB sensitivity (850 J m^{-2} dose) in vitro of a worldwide collection of 64 *P. syringae* strains representing 16 pathovars indicated that the most tolerant and most sensitive strains differed in percent survival by approximately 125-fold. We utilized Southern hybridization with an internal fragment of *rulAB* as a probe to show that 71.9% of the strains contained plasmid homologs of *rulAB* and that only two of the pathovars examined (actinidiae and syringae) included strains which did not contain *rulAB* hybridizing sequences. Strains which contained *rulAB* sequences were on average 5-50 fold more tolerant of UVB irradiation. Thus, the cloned *P. syringae rulAB* determinant was shown to confer significant levels of tolerance to UVB radiation both in vitro and in the natural habitat (phyllosphere) of the bacterium. Also, the phenotype of UVB tolerance and the plasmid-encoded *rulAB* genes were widely distributed among *P. syringae* pathovars. Our data suggest that tolerance to UVB radiation in *P. syringae* is an important component of ecological fitness in the phyllosphere.

Q-319. Characterization of Motor oil Utilizing Bacteria from Goucher Pond

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Twenty two bacterial isolates were obtained from oil slicks on Goucher Pond. The bacteria were isolated in minimal salt medium with 0.1 to 1% motor oil. Many isolates produced fluorescent pigments in King's B medium, which is limited in iron content. Nutritional and metabolic assays indicated that many of these bacteria belong to the fluorescent pseudomonads including Pseudomonas chlororaphis, P. fluorescens, P. putida, and P. viridiflava. One fluorescent isolate had the characteristics of Pseudomonas cepacia or P. gladioli which are not known to produce fluorescent pigments. One isolate, which was originally cocultivated with a fluorescent pseudomonad, was identified as Serratia ficaria. Except for S. ficaria, all isolates characterized thus far appear to produce rhamnolipids. Serratia ficaria alone did not survive in medium with motor oil as the sole carbon source. Some pseudomonad grew in motor oil as sole carbon source. However, its presence augmented the growth of other pseudomomdas in motor oil.

Q-320. Sulfur Cycling Mediates Calcium Carbonate Geochemistry in Modern Marine Stromatolites

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Modern marine stromatolites forming in Highborne Cay, Exumas (Bahamas), contain microbial mats dominated by *Schizothrix*. Although saturating concentrations of Ca^{2+} and CO_3^{2-} exist, microbes mediate $CaCO_3$ precipitation. Cyanobacterial photosynthesis in these stromatolites aids calcium carbonate precipitation by removal of H^+ through CO_2 use. Photorespiration and exopolymer production predominantly by oxygenic phototrophs fuel heterotrophic activity: aerobic respiration (approximately 60 mmol/cm^2.h) and sulfate reduction (SR; 1.2 mmol SO_4^{2-} /cm^2.h) are the dominant C-consuming processes. Aerobic microbial respiration and the combination of SR and H_2S oxidation both facilitate $CaCO_3$ dissolution through H^+ production. Aerobic respiration consumes much more C on an hourly basis, but diel fluctuating O_2 and H_2 depth profiles indicate that overall, SR consumes only slightly less (0.2-0.5) of the primary production. Moreover, due to low O_2 concentrations when SR rates are peaking, reoxidation of the H_2S formed is incomplete: both thiosulfate and polythionates are formed. The process of complete H_2S oxidation yields H^+. However, due to a low O_2 concentration late in the day and relatively high O_2 concentrations early in the following morning, a two-stage oxidation takes place: first, polythionates are formed from H_2S, creating alkalinity which coincides with $CaCO_3$ precipitation; secondly, oxidation of polythionates to sulfate yields acidity, resulting in dissolution, etc.

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Characterization of a Defined 2,3,5,6-Tetrachlorobiphenyl-*ortho*-Dechlorinating Microbial Community by Comparative Sequence Analysis of Genes Coding for 16S rRNA

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Defined microbial communities were developed by combining selective enrichment with molecular monitoring of total community genes coding for 16S rRNAs (16S rDNAs) to identify potential polychlorinated biphenyl (PCB)-dechlorinating anaerobes that *ortho* dechlorinate 2,3,5,6-tetrachlorobiphenyl. In enrichment cultures that contained a defined estuarine medium, three fatty acids, and sterile sediment, a *Clostridium* sp. was predominant in the absence of added PCB, but undescribed species in the δ subgroup of the class *Proteobacteria*, the low-G+C gram-positive subgroup, the *Thermotogales* subgroup, and a single species with sequence similarity to the deeply branching species *Dehalococcoides ethenogenes* were more predominant during active dechlorination of the PCB. Species with high sequence similarities to *Methanomicrobiales* and *Methanosarcinales* archaeal subgroups were predominant in both dechlorinating and nondechlorinating enrichment cultures. Deletion of sediment from PCB-dechlorinating enrichment cultures reduced the rate of dechlorination and the diversity of the community. Substitution of sodium acetate for the mixture of three fatty acids increased the rate of dechlorination, further reduced the community diversity, and caused a shift in the predominant species that included restriction fragment length polymorphism patterns not previously detected. Although PCB-dechlorinating cultures were methanogenic, inhibition of methanogenesis and elimination of the archaeal community by addition of bromoethanesulfonic acid only slightly inhibited dechlorination, indicating that the archaea were not required for *ortho* dechlorination of the congener. Deletion of *Clostridium* spp. from the community profile by addition of vancomycin only slightly reduced dechlorination. However, addition of sodium molybdate, an inhibitor of sulfate reduction, inhibited dechlorination and deleted selected species from the community profiles of the class *Bacteria*. With the exception of one 16S rDNA sequence that had the highest sequence similarity to the obligate perchloroethylene-dechlorinating *Dehalococcoides*, the 16S rDNA sequences associated with PCB *ortho* dechlorination had high sequence similarities to the δ, low-G+C gram-positive, and *Thermotogales* subgroups, which all include sulfur-, sulfate-, and/or iron(III)-respiring bacterial species.

The extensive industrial use of polychlorinated biphenyls (PCBs) during the 20th century has resulted in the release of an estimated several million pounds of PCBs into the environment (2). Due to the hydrophobicity and chemical stability of these compounds, PCBs ultimately accumulate in subsurface anaerobic sediments, where reductive dechlorination by anaerobic microorganisms is proposed to be an essential step in PCB degradation and detoxification (6). Although anaerobic reductive dechlorination has been documented in the environment and in the laboratory, attempts to identify and isolate anaerobic PCB-dechlorinating microbes by classical enrichment and isolation techniques have been unsuccessful (for a review, see reference 2). Isolation of anaerobic PCB-dechlorinating microbes has been hindered in part by the inability to maintain and sequentially transfer dechlorinating consortia in defined medium. May et al. (24) were the first to demonstrate that single colonies could be obtained by plating highly enriched PCB-dechlorinating enrichment cultures on agar-solidified media. Although two of the colonies exhibited *para* dechlorination activity when transferred back to liquid enrichment medium, the colonies contained a mixed community of microorganisms and dechlorination required the addition of sediment to the medium. More recently, highly enriched PCB-*ortho*-dechlorinating enrichment cultures were developed from Baltimore Harbor sediments in minimal media that contained sediments and a single congener (3) or Aroclor 1260 (37). These were the first confirmed reports of sustained *ortho* dechlorination of PCBs throughout sequential transfers in medium with estuarine sediments. Finally, Cutter et al. demonstrated that a consortium of PCB-*ortho*-dechlorinating anaerobes from Baltimore Harbor could be sequentially transferred and maintained in minimal medium without the addition of sterile sediment (9). With the ability to maintain PCB dechlorination in a completely defined medium, highly enriched PCB-dechlorinating consortia could be developed by sequential transfers in medium that contained the minimal growth requirements for dechlorinating species.

The current study identifies putative PCB-dechlorinating anaerobes in *ortho*-dechlorinating enrichment cultures by a comprehensive approach that combines traditional selective enrichment techniques with molecular monitoring (SEMM). Microbial consortia enriched for PCB *ortho* dechlorination in minimal medium were analyzed by comparative sequence analysis of genes coding for 16S rRNA (16S rDNA) amplified from total community DNAs. Protocols were developed for chro-

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mosomal DNA extraction from sediment, 16S rDNA amplification by PCR, cloning of partial 16S rDNA PCR fragments, screening by restriction fragment length polymorphism (RFLP) analysis, and DNA sequencing for comparative sequence analysis. By utilizing these techniques, shifts in the microbial community were monitored as the cultures were further enriched for PCB-dechlorinating anaerobes by elimination of undefined medium components (i.e., sediment), changes in carbon source, and addition of selective physiological inhibitors. The results presented herein demonstrate the applicability of the SEMM approach for the selection and monitoring of highly defined PCB-dechlorinating microbial consortia.

MATERIALS AND METHODS

Enrichment cultures. Enrichment cultures were initiated as described previously (9). Briefly, sediment samples collected from the Northwest Branch of Baltimore Harbor, Baltimore, Md. (39°16.8'N, 76°36.1'W), were used to inoculate sterile, anaerobic estuarine salts medium that did not contain added sulfate to a final concentration of 5% (dry wt/vol). Where indicated, sodium acetate, alone or with sodium propionate and butyrate, was added to a final concentration of 2.5 mM (each). The congener 2,3,5,6-tetrachlorobiphenyl (2,3,5,6-CB; AccuStandard, Inc., New Haven, Conn.) was solubilized in acetone and added to a final concentration of 173 μM. For the inhibitor studies, bromoethanesulfonic acid (BES), vancomycin, and sodium molybdate were dissolved in deionized water, filter sterilized, and added to final concentrations of 3 mM, 100 μg/ml, and 20 mM, respectively. All cultures were incubated in the dark at 30°C. PCBs were extracted and analyzed by gas chromatography coupled with an electron capture detector using a 16-point standard curve for each congener as described previously (3).

Extraction of genomic DNA. The methods described herein for the phylogenetic analysis of the enrichment cultures are slightly modified from those described previously (13). Depending upon the culture turbidity, between 1 and 10 ml of culture was anaerobically withdrawn and utilized for extraction of bulk genomic DNA (final yield, greater than 100 ng as estimated by visualization on an agarose gel stained with ethidium bromide). The culture sample was centrifuged, and the cell and sediment pellet was resuspended in 250 μl of sterile TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). The resuspended pellet was added to a 2.2-ml screw-cap conical tube that contained 2.5 g of autoclaved zirconia-silica beads (0.1 mm), and 250 μl each of sodium phosphate buffer (0.1 M, pH 8.0) and TS-SDS buffer (0.1 M NaCl, 0.5 M Tris [pH 8.0], 10% [wt/vol] sodium dodecyl sulfate). The sample was cooled on ice for 10 min and then homogenized for 5 min with a Mini-Bead Beater (Biospec, Bartlesville, Okla.) at 4°C to lyse cells. Debris was removed by centrifugation for 5 min at 14,000 × *g*. Crude DNA in the supernatant was purified twice with equal volumes of trissaturated phenol and chloroform-isoamyl alcohol (24:1), followed by extraction with an equal volume of chloroform. Approximately 200 μl of Phase-Lock gel (5 Prime-3 Prime, Inc., Boulder, Colo.) was utilized to promote separation of the phases and allow easier visualization of the interface. The decanted aqueous phase was diluted to 1 ml with sterile deionized water. Humic acids, which inhibit PCR (32, 34), were extracted from nucleic acids by addition of 0.125 g of insoluble polyvinylpolypyrrolidone (Sigma, St. Louis, Mo.) to the 1 ml of diluted crude DNA extract (17, 30). The polyvinylpolypyrrolidone was removed by centrifugation for 5 min at 14,000 × *g*, and the chromosomal DNA was recovered by precipitation with an equal volume of isopropanol at −20°C. The DNA was pelleted by centrifugation, and then the pellet was washed with 70% ethanol and centrifuged again at high speed. The supernatant was discarded, and the DNA was dried under vacuum for 5 min. Further removal of humic acids was achieved by electrophoresis of the DNA extract in a 1.3% low-melting-point agarose gel (Fisher Scientific, Fairlawn, N.J.) containing 2% soluble polyvinylpyrrolidone (40). The chromosomal DNA band was excised from the gel and recovered with a Promega Wizard PCR Prep Kit (Promega, Madison, Wis.) in accordance with the manufacturer's instructions.

PCR amplification and cloning. PCR was utilized to amplify bacterial and archaeal 16S rDNAs from the mixed community of genomic DNAs. Universal primers 519F (5'-CAG CA/CG CCG CGG TAA TA/TC-3') and 1406R (5'-ACG GGC GGT GTG TA/GC-3') were utilized for the amplification of bacterial 16S rDNAs (21). Archaeal 16S rDNAs were amplified with specific archaeal primers 21F (5'-TTC CGG TTG ATC CYG CCG GA-3') and 958R (5'-TCC GGC GTT GAM TCC AAT T-3') (11). All PCR amplifications were performed by using the GeneAmp PCR kit with *Taq* DNA polymerase (Perkin Elmer, Inc.) in a PTC200 thermal cycler (MJ Research, Watertown, Mass.). Conditions for PCR were as follows: an initial denaturation step of 1.5 min at 94°C; 30 amplification cycles of denaturation (30 s at 94°C), annealing (30 s at 55°C), and elongation (30 s at 72°C); and a final extension step of 5 min at 72°C. The PCR products were purified by utilizing the QIAquick PCR purification kit (Qiagen, Inc., Chatsworth, Calif.). Plasmid libraries for both domains were generated by ligating 2 μl of purified PCR fragments into the pCRII vector (Invitrogen, Carlsbad, Calif.) in accordance with the manufacturer's instructions. The ligation reactions were transformed into the *Escherichia coli* INVαF' competent cells supplied with the Invitrogen Original TA Cloning Kit.

Library screening. Ninety-six randomly chosen clones were selected from colonies and grown overnight in Luria broth with kanamycin (100 μg/ml). The partial 16S rDNA fragments were amplified directly from 2 μl of an overnight-grown Luria broth culture added to 48 μl of PCR mixture using the following PCR conditions: 1 cycle of 3 min at 95°C; 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension step of 72°C for 5 min. Subsequently, the PCR products were digested separately with the restriction endonucleases *Hae*III and *Hha*I (New England Biolabs, Inc., Beverly, Mass.). The restriction digests were electrophoresed in a 3% Trevi-Gel (TreviGen, Gaithersburg, Md.) and visualized with SYBR Green I nucleic acid gel stain (Molecular Bio-Probes, Eugene, Oreg.) by using a Fluoroimager (Molecular Dynamics, Sunnyvale, Calif.). Clones were categorized according to their distinct RFLPs.

Sequencing and analysis. At least two representative clones for each unique RFLP were sequenced for comparative phylogenetic analysis. Plasmid DNA was purified with the Qiagen Plasmid Mini Kit (Qiagen, Inc.), and the sequence was determined after dye terminator cycle sequencing on an ABI 373 Automated Sequencer (Applied Biosystems, Foster City, Calif.). Initially, the clones were sequenced from the flanking 5' end with a T7 sequencing primer and from the flanking 3' end with an M13 reverse sequencing primer, both located on the pCRII vector, to obtain the complete fragment sequence.

Sequences were analyzed with the National Center for Biotechnology Information basic local alignment search tool via the BLASTN program (1) and the SIM_RANK program of the Ribosomal Database Project (28).

Chimeric sequence evaluation. Screening methods similar to those described previously by Snaidr et al. (29) were utilized for chimera screening. First, the sequences were manually aligned and then analyzed by using a software package that takes into account misalignments in secondary structure that could result from chimeras (7). Second, short sequences (~300 bp) of both the 16S rDNA 5' and 3' flanking regions were then submitted to both the BLASTN and SIM_RANK programs for comparative phylogenetic analysis of whole and partial gene sequences. Third, partial sequences were evaluated with the Check_Chimera program of the Ribosomal Database Project. To further minimize chimera formation, high-molecular-weight genomic DNA and PCR products were size fractionated in agarose gels prior to library construction. In addition, both bacterial and archaeal clone libraries were generated and screened from three replicate PCRs.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences used to generate a phylogenetic tree are as follows: *Clostridium litorale*, X77845; *Dehalobacter restrictus*, U84497; *Dehalococcoides ethenogenes*, AF004928; *Desulfitobacterium dehalogenans*, L28946; *Desulfitobacterium frappieri*, U40078; *Desulfobacter postgatei*, M26633; *Desulfomonile tiedjei*, M26635; *Desulfonema ishimotoei*, U45992; *Desulfosarcina variabilis*, M34407; *Desulfothiovibrio peptidovorans*, U52817; *Desulfotomaculum orientis*, M34417; *Desulfovibrio desulfuricans*, M34113; *Desulfuromonas acetexigens*, U23140; *Desulfuromusa succinoxidans*, X79415; *Fervidobacterium nodosum*, M59177; *Geobacter metallireducens*, L07834; *Geotoga petraea*, L10658; *Pelobacter propionicus*, X70954; *Petrotoga miotherma*, L10657; *Syntrophospora bryantii*, M26491; *Syntrophus gentianae*, X85132; *Thermoanaerobacter brockii*, L09165; *Thermosipho africanus*, M83140; *Thermotoga maritima*, M21774.

Sequences of the partial 16S rDNA clones exhibiting RFLP types 1, 4, 5, 11, 15, 17, 24, 25, 40, 105, 108, 109, and 144 were submitted to GenBank under accession no. AF058000 to AF058012, respectively.

RESULTS

Effects of PCB on community profiles. Selective enrichment techniques were used to establish *ortho*-dechlorinating enrichment cultures. Concomitantly, the cultures were monitored by screening the 16S rDNA community for putative PCB-*ortho*-dechlorinating microorganisms within these enrichment cultures. The diversity of the microbial community was minimized from the outset by the use of a minimal estuarine medium that contained sterilized Baltimore Harbor sediments. Further, the enrichment cultures were incubated with a single PCB congener, 2,3,5,6-CB, to facilitate monitoring of the rate and extent of dechlorination and to select for congener-specific dechlorinating organisms that were capable of reductively dechlorinating the parent congener and its trichlorinated intermediate (3).

Enrichment cultures that exhibited *ortho* dechlorination of 2,3,5,6-CB were generated by three sequential transfers (10% inoculum) of Baltimore Harbor sediments in estuarine medium supplemented with a mixture of three fatty acids: propionate, butyrate, and acetate (3, 9). Following the third sequential transfer, the only dechlorination pathway observed for these cultures, *ortho* dechlorination of 2,3,5,6-CB (Fig. 1A,

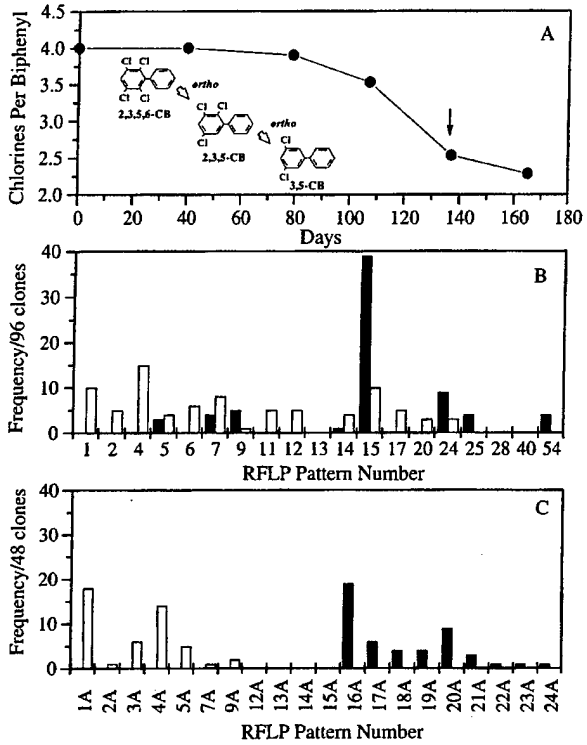

FIG. 1. (A) Rate of chlorine removal from 2,3,5,6-CB by enrichment cultures containing 0.1% Baltimore Harbor sediment. The dechlorination pathway of 2,3,5,6-CB by *ortho*-dechlorinating enrichment cultures is shown in the inset. (B) Community profiles of bacterial 16S rDNA clones from Baltimore Harbor enrichment cultures incubated with (□) and without (■) 2,3,5,6-CB. Samples for phylogenetic analysis were taken at day 137, as indicated for panel A. Both enrichment cultures were amended with a mixture of three fatty acids as carbon sources. (C) Community profiles of archaeal 16S rDNA clones from Baltimore Harbor enrichment cultures incubated with (□) and without (■) 2,3,5,6-CB.

inset), was observed in the PCB-containing culture after 79 days and achieved a maximum rate after 107 days (Fig. 1A). Approximately 75% of the parent congener was converted to 3,5-CB after 160 days. Duplicate enrichment cultures that did not contain a PCB were maintained and sequentially transferred concurrently with the PCB-dechlorinating enrichment cultures. Both dechlorinating and nondechlorinating enrichment cultures were methanogenic.

Community profiles analyzed at 137 days after the third sequential transfer of dechlorinating and nondechlorinating enrichment cultures are shown in Fig. 1B. Sixteen predominant RFLP types were identified in the cultures, and 16S rDNA fragments from two representative clones for each pattern were subjected to comparative sequence analysis. Eight RFLP types, 1, 2, 4, 6, 11, 12, 17, and 20, were detected exclusively in cultures that contained the PCB congeners. RFLP type 4, the most predominant clone, accounting for 30% of the selected clones, showed the highest sequence similarity to the δ subgroup (Table 1). RFLP type 1, the second most predominant clone, accounted for 20% of the selected clones and showed the highest sequence similarity to the *Thermotogales* subgroup. Of the remaining clones, RFLP types 11 and 12 had the highest sequence similarity to the low-G+C gram-positive subgroup, RFLP types 4, 6, and 20 had the highest sequence homology to members of the δ subgroup, and RFLP type 17 exhibited the highest sequence similarity to the deeply branching species *Dehalococcoides ethenogenes* (25). Only one representative clone with RFLP type 6 was identified because the partial 16S rDNA insert was unstable and often lost from the vector prior to sequencing.

RFLP types 7 and 14 showed the highest sequence similarity to the low-G+C gram-positive subgroup. Both patterns were detected in the presence and absence of a PCB but increased significantly (≥50%) in medium that contained a PCB. The remaining clones, which had high sequence similarity to members of the δ subgroup (RFLP type 25) and the low-G+C gram-positive subgroup (RFLP types 5, 9, 15, 24, and 54), were either detected at similar frequencies in both cultures, increased in the frequency of detection relative to one another, or detected only in the PCB-free culture. The results suggest that species represented by the latter clones do not have a significant role in PCB *ortho* dechlorination.

The community profiles of methanogenic archaea enriched in the presence and absence of a PCB differed significantly (Fig. 1C). Seven predominant RFLP types were detected in the actively dechlorinating culture. RFLP types 1A, 4A, and 5A had the highest sequence similarity to the *Methanosarcinales* subgroup, whereas RFLP types 2A, 3A, 7A, and 9A had the highest sequence similarity to the *Methanomicrobiales* subgroup (Table 2). Conversely, none of the clones detected in the presence of a PCB were detected in the PCB-free enrichment culture. RFLP types 16A, 19A, 20A, 21A, 22A, and 24A had the highest sequence similarity to the *Methanosarcinales* subgroup, and the remaining clones, with RFLP types 17A, 18A, and 23A, had the highest similarity to the *Methanomicrobiales* subgroup. Although the community profiles differed in the absence and presence of a PCB congener, both cultures exhibited similar distributions of species belonging to the autotrophic, hydrogen-utilizing order *Methanomicrobiales* and the aceticlastic and methylotrophic order *Methanosarcinales*. This preliminary

TABLE 1. Phylogenetic affiliations of predominant RFLP types from PCB-*ortho*-dechlorinating enrichment cultures based on bacterial 16S rRNA gene sequences

RFLP type	Closest phylogenetic relative	% Similarity to closest relative
1	*Thermotoga maritima*	85
2	*Bacteroides eggerthii*	89
4	*Desulfosarcina variabilis*	93
5	*Desulfothiovibrio peptidovorans*	87
6	*Desulfuromonas thiophila*	94
7	*Clostridium litorale*	91
9	*Desulfonema magnum*	82
11	*Syntrophospora bryantii*	94
12	Unidentified oil field bacterium	75
15	*Clostridium litorale*	99
17	*Dehalococcoides ethenogenes*	89
20	*Pelobacter acidigallici*	86
24	*Acholeplasma laidlawii*	84
25	*Desulfonema magnum*	94
28	*Desulfovibrio caledoniensis*	95
40	*Syntrophus gentianae*	94
54	*Clostridium litorale*	84
105	*Desulfuromonas thiophila*	96
108	*Desulfuromonas acetexigens*	99
109	*Desulfovibrio* sp.	92
116	*Desulfovibrio* sp.	86
130	Uncultured eubacterium	89
138	Unidentified low-G+C gram-positive sp.	96
144	*Desulfovibrio* sp. strain B650	98
146	*Desulfovibrio* sp.	91

TABLE 2. Phylogenetic affiliations of predominant RFLP types from PCB-*ortho*-dechlorinating enrichment cultures based on archaeal 16S rRNA gene sequences

RFLP type	Closest phylogenetic relative	% Similarity to closest relative
1A	*Methanosaeta concilii*	91
2A	*Methanoculleus marisnigri*	90
3A	*Methanoplanus limicola*	90
4A	*Methanohalophilus mahii*	87
5A	*Methanohalobium evestigatum*	81
7A	*Methanogenium organophilum*	96
9A	*Methanospirillum hungatei*	87
16A	*Methanosaeta concilii*	99
17A	*Methanoplanus petrolearius*	94
18A	*Methanogenium organophilim*	96
19A	*Methanosaeta concilii*	96
20A	*Methanohalophilus mahii*	86
21A	*Methanosaeta concilii*	96
22A	*Methanosaeta concilii*	99
23A	*Methanoplanus limicola*	92
24A	*Methanosaeta concilii*	99

characterization represented a baseline community profile for the PCB-dechlorinating and nondechlorinating enrichment cultures.

Effects of Baltimore Harbor sediment on *ortho*-dechlorinating consortia. To eliminate the effects of putative alternative electron acceptors (e.g., humic acids, SO_4^{2-}, Fe^{2+}) and undefined nutrients that may be present in Baltimore Harbor sediments, PCB-dechlorinating enrichment cultures were sequentially transferred in completely defined estuarine medium that contained 2,3,5,6-CB and three fatty acids as carbon sources without the addition of sterile sediments (9). After four sequential transfers in the absence of sediments, dechlorination of 2,3,5,6-CB was detected after an extensive lag period (>100 days) and the congener was completely transformed to 3,5-CB after 240 days (Fig. 2A). Methane production was observed in the sediment-free enrichment cultures.

Community profiles were compared before and after the onset of dechlorination in the fourth sequential enrichment culture transfer in defined medium (Fig. 2B). Of the 14 predominant RFLP types previously detected in PCB-dechlorinating cultures with sediment, 10 were detected in the sediment-free cultures. As observed in the previous cultures, RFLP type 1 was the predominant species, accounting for 36% of the clones detected. Of the seven remaining RFLP types that appeared exclusively in the PCB-dechlorinating enrichment culture with sediment, only four were detected in the absence of sediment (RFLP types 4, 6, 11, and 17) and only the relative detection frequencies of RFLP type 5 increased significantly with the onset of dechlorination. The absence of RFLP types 2, 9, 12, 14, 20, and 54 indicated that these species were diluted out to undetectable levels after sediment was deleted. Although this observation suggests that the latter species are not required for *ortho* dechlorination of 2,3,5,6-CB, it does not rule out the possibility that they are capable of dechlorination but lacked specific growth factors provided by the sediments. The three remaining clones, RFLP types 28, 40 (δ subgroup), and 13 (low-G+C gram-positive subgroup), were not observed previously in medium that contained sediment but were selectively enriched in the absence of sediment.

Overall, the most predominant members of the methanogenic archaeal community did not change significantly with the onset of dechlorination in the sediment-free enrichment cultures, as indicated in Fig. 2C, and all were observed in previous cultures with sediment and the PCB congener. RFLP types 4A, 12A, and 14A were detected only after dechlorination was observed in the enrichment. RFLP types 3A, 5A, and 13A were detected both in the preactive and active cultures. RFLP type 15A was detected only in the absence of dechlorination. RFLP type 5A, the most predominant clone, had the highest sequence homology to members of the order *Methanosarcinales*, whereas the second most predominant clone, RFLP 3A, had the highest homology to members of the order *Methanomicrobiales*.

Effects of carbon source on *ortho*-dechlorinating consortia. PCB-dechlorinating enrichment cultures grown with three fatty acids were sequentially transferred into defined estuarine medium that contained 2,3,5,6-CB and sediment with sodium acetate as the sole electron donor to minimize community diversity further. After three sequential transfers, dechlorination was detected within 28 days and the congener was completely transformed to 3,5-CB after 85 days (Fig. 3). Growth rates were not measured in cultures that contained sediment due to turbidity caused by the particles. However, enrichment cultures that contained sodium acetate had higher dechlorination rates than cultures that contained a mixture of three fatty acids. Cultures were methanogenic with sodium acetate.

Community profiles were determined after three sequential transfers of the enrichment cultures with 2,3,5,6-CB and so-

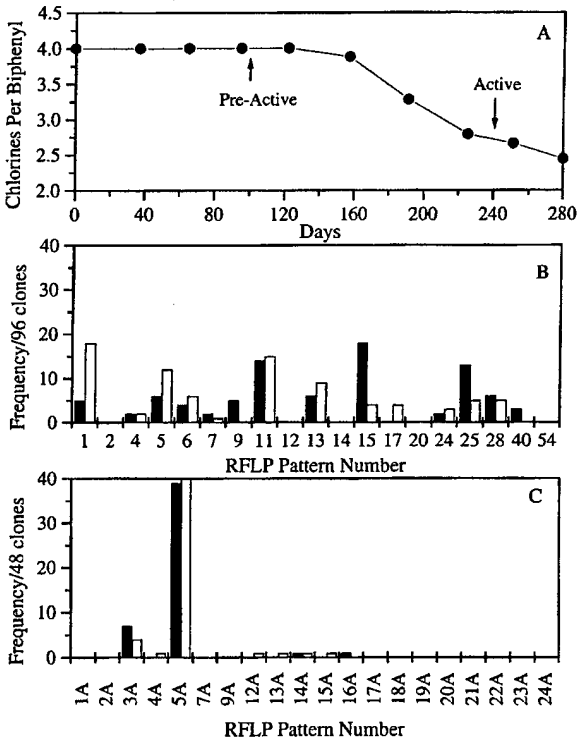

FIG. 2. (A) Reductive dechlorination of 2,3,5,6-CB in sediment-free Baltimore Harbor enrichment cultures with a mixture of three fatty acids as carbon sources. Sediment was removed by dilution after four sequential transfers. The enrichment culture was sampled for phylogenetic analysis prior to the onset of dechlorination (preactive, day 102) and during *ortho* dechlorination (active, day 240). (B) Community profiles of bacterial 16S rDNA clones from sediment-free Baltimore Harbor enrichment cultures prior to (■) and following (□) the onset of *ortho* dechlorination. (C) Community profiles of archaeal 16S rDNA clones from sediment-free Baltimore Harbor enrichment cultures prior to (■) and following (□) the onset of *ortho* dechlorination.

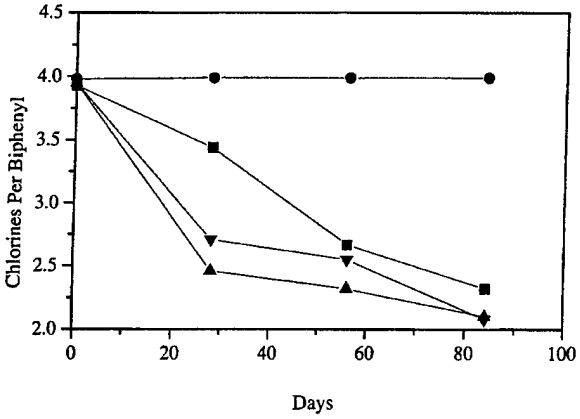

FIG. 3. Dechlorination rates of Baltimore Harbor cultures treated with physiological inhibitors. Symbols: ▲, no inhibitor; ■, 3 mM BES; ●, 20 mM sodium molybdate; ▼, 100-μg/ml vancomycin.

dium acetate (Fig. 4A). Only 6 RFLP types, 6, 7, 15, 17, 24, and 40, of the 19 predominant RFLP types detected in the previous cultures that contained fatty acids were detected in cultures that contained only acetate as an electron donor. Interestingly, RFLP types 4, 5, 11, 13, 25, and 28, which were predominant in cultures that contained a mixture of fatty acids that included sodium acetate, were not detected in dechlorinating enrichment cultures grown with sodium acetate alone. These results suggest that growth of the latter species was linked to butyrate or propionate catabolism. The shift to acetate resulted in a significant overall change in the community. The most predominant RFLP types (105, 108, 109, and 116; frequency, ≥2/96 clones) detected in enrichment cultures containing sodium acetate were not detected previously, indicating that their growth may be linked specifically to acetate. All of the predominant RFLP types belonged to the δ subgroup.

Effects of selective inhibitors on *ortho*-dechlorinating consortia. To further reduce community diversity and select for microbial species linked to *ortho* dechlorination of 2,3,5,6-CB with sodium acetate as the growth substrate, enrichment cultures were transferred into medium that contained physiological inhibitors. The inhibitors included BES, which selectively inhibits the methanogenic archaea (16); sodium molybdate, an analogue of sulfate, which selectively inhibits sulfate-reducing bacteria (31); and vancomycin, which selectively inhibits gram-positive bacteria by inhibiting biosynthesis of the cell wall peptidoglycan (27). Active cultures were transferred to medium that contained the selected physiological inhibitor and then sampled for analysis of the 16S rDNA community profile after the onset of dechlorination.

The addition of BES only slightly inhibited the rate of dechlorination, and nearly complete dechlorination of 2,3,5,6-CB to 3,5-CB occurred within 85 days (Fig. 3). The bacterial diversity and relative numbers of bacterial species in the BES-treated culture closely resembled those in untreated control cultures (Fig. 4A and B). Seven previously undescribed RFLP types were detected, but only RFLP type 130 (low-G+C gram-positive subgroup) was predominant at frequencies of ≥2/96 clones sampled. However, methanogenesis did not occur and archaeal rDNA was not detected by PCR, indicating that the methanogenic archaea were not required for *ortho* dechlorination of 2,3,5,6-CB to 2,3,5-CB and 3,5-CB with sodium acetate.

As expected, vancomycin caused a more significant shift in the bacterial community than BES (Fig. 4C). Interestingly, vancomycin, like BES, also inhibited methanogenesis and precluded detection of archaeal rDNA by PCR, confirming that the methanogenic archaea were not required for *ortho* dechlorination of 2,3,5,6-CB with sodium acetate. Five RFLP types, 6, 7, 17, 24, and 105, were detected previously in PCB-dechlorinating cultures that did not contain an inhibitor. Of the 10 RFLP types not detected previously, the two most predominant (frequency, ≥2/96 clones), 144 and 146, were most closely related to the δ subgroup.

The addition of sodium molybdate (final concentrations of 2 and 20 mM) completely inhibited dechlorination and inhibited methanogenesis of 2,3,5,6-CB (Fig. 3). Furthermore, the genomic yield of this culture was approximately 10-fold lower than that of the previous cultures, and the bacterial diversity was significantly reduced (Fig. 4D). As expected, RFLP types 40, 105, 108, 109, and 116, which had sequence similarity to the δ subgroup, were not detected in the molybdate culture. However, the relative detection frequency of RFLP type 6, which is also phylogenetically related to the δ subgroup, was similar to that of the positive control, along with low-G+C gram-positive RFLP types 7, 15, and 24. RFLP type 138 (low-G+C gram-

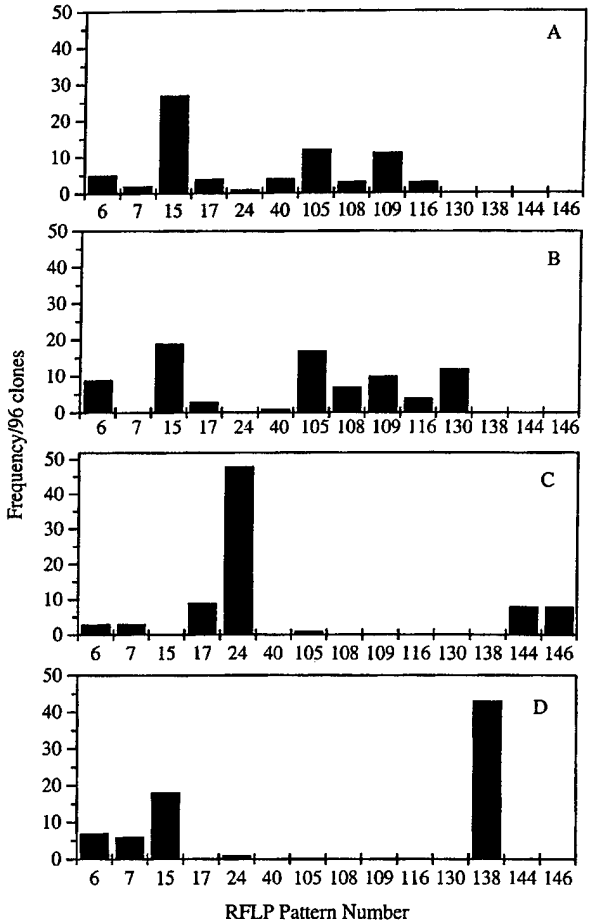

FIG. 4. Effects of physiological inhibitors on community profiles of Baltimore Harbor enrichment cultures enriched with 2,3,5,6-CB, acetate, and 0.1% Baltimore Harbor sediment. Panels: A, no inhibitor; B, 3 mM BES; C, 100-μg/ml vancomycin; D, 20 mM sodium molybdate.

positive subgroup) was detected only in this culture and, therefore, was unlikely to represent an *ortho*-dechlorinating species.

DISCUSSION

Molecular screening of the 16S rDNAs from the total community of genomic DNAs was used to characterize microbial consortia in PCB-*ortho*-dechlorinating enrichment cultures without isolation of heretofore unculturable dechlorinating species. Bias can be introduced at various stages in the protocol, particularly during cell lysis and PCR amplification. Therefore, to minimize screening bias, a physical cell lysis method, bead mill homogenization, was used to effectively lyse all cell types, including those most recalcitrant to physical and enzymatic treatments (22, 26). To minimize PCR bias, separate primers were used for bacterial and archaeal phylogenetic domains. The primers were tested with Baltimore Harbor enrichment cultures and determined empirically to yield greater community diversity than other "universal" primers previously described (data not shown). In addition, PCR parameters, including use of a denaturant (formamide), temperature, and ion concentration, were optimized to yield maximum diversity in the community profiles of Baltimore Harbor enrichment cultures. Other factors, such as species-specific 16S rDNA copy number and PCR bias for a low-G+C template, also affect the quantitative assessment of microbial communities (14), and as a result, this approach can provide only an estimate of the actual abundance of microorganisms in each enrichment. In the current study, all enrichment cultures were sequentially transferred from the same inoculum source and grown under similar conditions. Throughout the study, community profile comparisons of duplicate cultures and of sequential transfers of identical treatments were reproducible (data not shown). Therefore, it was possible to determine whether an individual species was associated with PCB dechlorination by assaying for the coexistence or mutual exclusion of its RFLP type with dechlorination after treatment with physiological inhibitors. By monitoring the rates of dechlorination and relative frequencies of detection of specific RFLP types associated with PCB dechlorination, this approach was used to establish a highly defined PCB-*ortho*-dechlorinating community and to monitor the effects of sequential culture transfers and treatments on specific community members.

Previous attempts to identify and isolate anaerobic PCB dechlorinators by selective enrichment and isolation techniques have been unsuccessful (2). The failure to identify these species is likely due to the development of previous enrichment cultures in complex, undefined medium, which resulted in selection for faster-growing, non-PCB-dechlorinating microorganisms that likely outcompete PCB dechlorinators. By using the SEMM approach, conditions were developed that would maintain cultures of PCB-dechlorinating consortia indefinitely in a defined minimal medium. While other molecular approaches have been described for the isolation of bacteria from the environment (19, 23, 33), this is the first reported application of a molecular approach for the development of a defined PCB-dechlorinating consortium in a minimal medium. By reducing the medium complexity, the community diversity in a PCB-dechlorinating consortium was systematically reduced with the addition of medium components and physiological inhibitors that selectively promoted the growth of species involved in *ortho* dechlorination of 2,3,5,6-CB. Screening of the microbial communities by RFLP of PCR-amplified 16S rDNA as the cultures were selectively enriched provided a means for effectively monitoring the effects of treatments on individual species and, by a process of elimination, enabled us to identify species that are most likely to catalyze PCB dechlorination. In addition, the phylogeny of individual RFLP types was determined by comparative sequence analysis of the PCR-amplified 16S rDNA fragments (Fig. 5).

By sequentially transferring cultures in both the presence and the absence of 2,3,5,6-CB, species that had a selective growth advantage with the congener were enriched, as indicated by differences in the community profiles. However, several RFLP types were present under both culture conditions, indicating that these species utilized alternative electron acceptors to PCB for growth. Possible mechanisms included (i) methanogenic carbon dioxide reduction by hydrogen-utilizing methanogens via interspecies hydrogen exchange with propionate- and butyrate-utilizing acetogens or acetate-dismutating species, which include low-G+C gram-positive species such as clostridia and members of the δ subgroup; (ii) dismutation of acetate by aceticlastic methanogens; (iii) fatty acid oxidation with unknown dissimilatory electron acceptors in sediment; and (iv) fatty acid oxidation with PCB as a dissimilatory electron acceptor.

To further reduce selection to growth-linked or cometabolic PCB dechlorination, enrichment cultures were initiated and sequentially transferred into totally defined sediment-free medium. Although the medium complexity was reduced, the overall community diversity was reduced only slightly and the same phylogenetic groups (the δ, low-G+C gram-positive, *Thermotogales*, and *Dehalococcoides* subgroups) were detected, indicating that most species from the initial enrichment cultures adapted to growth without sediments. Past reports have indicated that sediments were required in order to maintain microbially mediated PCB-dechlorinating activity through sequential transfers, and several possible roles for sediment in the dechlorination process are discussed by Cutter et al. (9) and Boyle et al. (5). By developing a microbial community adapted to growth in defined medium, it was possible to further reduce the complexity of the *ortho*-dechlorinating community systematically by eliminating or substituting components.

The influence of the carbon source on the community of PCB-dechlorinating enrichment cultures was investigated. Changing the carbon source from a mixture of butyrate, propionate, and acetate to acetate as the sole electron donor caused a dramatic shift in the microbial community. Although the growth rates observed in enrichment cultures with the mixture of fatty acids were greater than rates observed in cultures with acetate alone, the dechlorination rate was greater in enrichment cultures that contained acetate alone. It is well documented that enrichment conditions, choice of PCB congener, and source of inoculum can influence dechlorinating activities (2). However, this is the first confirmed report of the influence of an electron donor on the community profile of a PCB-dechlorinating enrichment culture.

The overall results of this study show that the defined growth conditions supported the growth of only four phylogenetic subgroups among the bacteria, i.e., the δ, low-G+C gram-positive, and *Thermotogales* subgroups and a single species near the deeply branching species *D. ethenogenes*, and two phylogenetic subgroups among the archaea, i.e., the H_2-CO_2 utilizing *Methanomicrobiales* subgroup and the methylotrophic and aceticlastic *Methanosarcinales* subgroup (Fig. 5). The detection of the H_2-CO_2-utilizing methanogens indicates that hydrogen was likely generated by fatty acid-oxidizing acetogenic bacteria. This conclusion is supported by the observation that H_2-CO_2-utilizing *Methanomicrobiales* and methylotrophic and aceticlastic *Methanosarcinales* subgroup species are evenly distributed when enrichment cultures are grown on a mixture of fatty

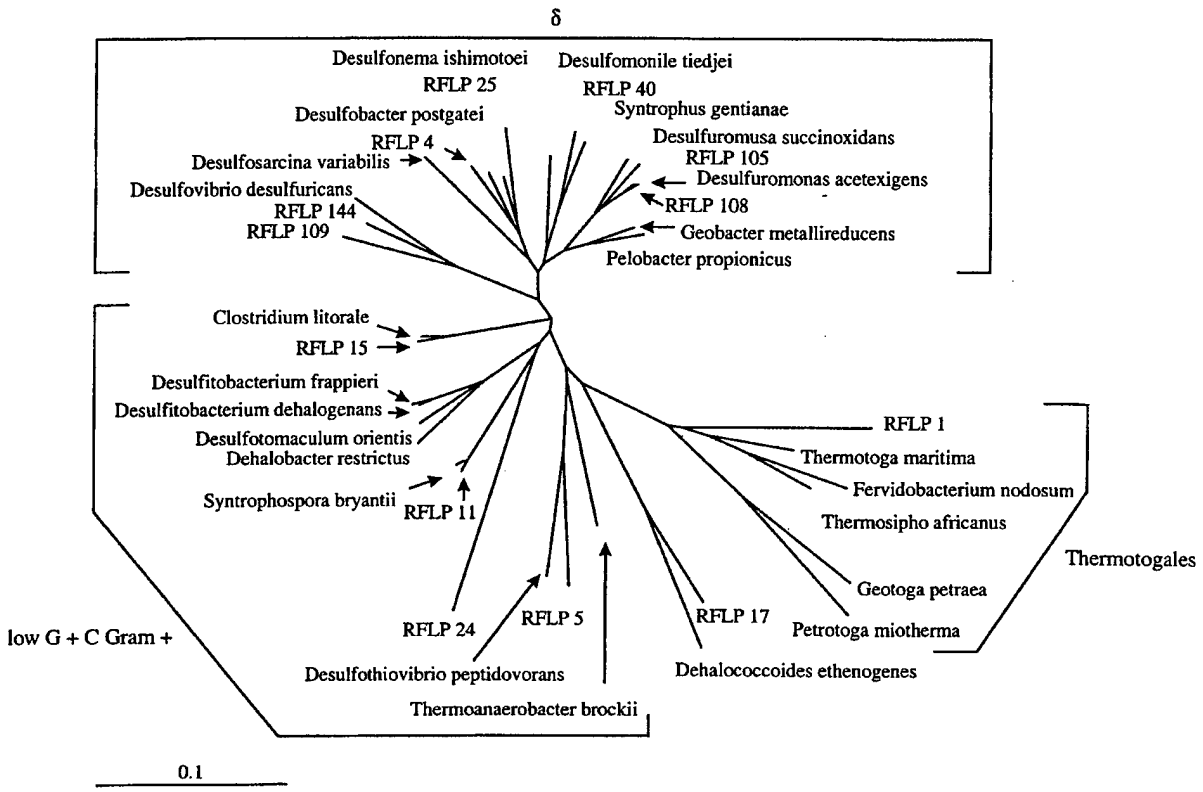

FIG. 5. Phylogenetic tree inferred from comparative sequence analyses of partial 16S rDNA sequences from several predominant clones obtained from PCB-*ortho*-dechlorinating enrichment cultures. For construction of a phylogenetic tree, approximately 890-bp segments of selected sequences were aligned manually with a collection of known bacterial 16S rRDAs (for nucleotide sequence accession numbers, see Materials and Methods) obtained from the GenBank database by using software described by Chun (7). Evolutionary distances, expressed as estimated changes per 100 nucleotides, were calculated from the percentages of similarity by using the correction of Jukes and Cantor (18). A dendogram was constructed with PHYLIP based on the unweighted pair group method with arithmetic averages (15). The bar represents 0.1 U of evolutionary distance.

acids, but *Methanosarcinales* species become most predominant with acetate only. However, dechlorination was observed when methanogenesis and growth of all methanogenic archaea were inhibited by BES, indicating that methanogenic archaea are not required for acetate-mediated *ortho* dechlorination of 2,3,5,6-CB. The slight inhibition of dechlorination with BES treatment likely resulted from nonspecific inhibition of bacterial species that were involved in dechlorination. This conclusion is further supported by the observation that vancomycin treatment also inhibited methanogenesis and methanogen growth but had only a slight effect on the rate of dechlorination. A report by May et al. indicated that colonies of PCB-enriched consortia plated on solidified media *para* and/or *meta* dechlorinated 2,3,4-CB and 2,4,5-CB in the absence of methanogenesis (24). In contrast, the same cultures lost the ability to dechlorinate 2,5,3′,4′-CB and 3,4,2′-CB concurrently with the loss of methanogenic activity. Likewise, Ye et al. (38) reported that methanogenesis occurred concurrently with process H (*meta*, *para*) dechlorination of Aroclor 1242 but that process M (*meta*) dechlorination occurred in the absence of methanogenesis. Results of the current study show that *ortho* dechlorination of 2,3,5,6-CB is catalyzed in the absence of methanogenesis. These results, in conjunction with previous reports on *para* and *meta* dechlorination of individual congeners and Aroclors, support the hypothesis that different phylogenetic groups of bacteria and archaea dechlorinate selected PCB congeners.

RFLP type 15, which had high sequence similarity to *Clostridium* sp., was inhibited by the addition of vancomycin but not by molybdate. Reduction in the relative abundance of RFLP type 15 by the addition of vancomycin or by the removal of sediment did not affect the rate of removal of *ortho* chlorines from 2,3,5,6-CB, which suggests that RFLP type 15 does not have a role in dechlorination. Following pasteurization (80°C for 1 h) of cultures containing fatty acids and sediment, *ortho* dechlorination ceased, further supporting the conclusion that spore-forming microbes such as *Clostridium* spp. are not responsible for *ortho* dechlorination. In contrast, *para* and *meta* dechlorination of Aroclor 1242 by Hudson River sediments was shown to be resistant to pasteurization (36). Davenport et al. have reported that archaeal and clostridial 16S sequences are predominant in microcosms that *meta* and *para* dechlorinate 2′,3,4-CB (10). However, neither of the latter two studies reported *ortho* dechlorination, which further supports the hypothesis that different species exhibit congener specificity.

Species most frequently associated with *ortho* dechlorination of 2,3,5,6-CB in the Baltimore Harbor enrichment cultures had high sequence similarities to described species of dissimilatory sulfur- and sulfate/iron-reducing bacteria. In the presence of molybdate, *ortho* dechlorination of 2,3,5,6-CB was inhibited. Further, with the exception of one species, all of the 16S rDNA clones frequently associated with actively dechlorinating cultures cluster with the sulfate/iron-dissimilating δ subgroup or

the elemental sulfur/thiosulfate/sulfite-dissimilating low-G+C gram-positive and *Thermotogales* subgroups. Ye proposed that spore-forming dissimilatory sulfate-reducing bacteria were responsible for process M (*meta*) dechlorination, since pasteurization and ethanol treatment did not inhibit dechlorinating activity in freshwater cultures but addition of molybdate did inhibit activity (39). In addition, described species that reductively dechlorinate aromatic or aliphatic compounds also cluster with sulfate or sulfur/iron reducers in the δ subgroup (e.g., *Desulfomonile tiedjei*, *Pelobacter* sp. TT4B strain 2CP1) and with the sulfur/thiosulfate/sulfite reducers in the low-G+C gram-positive subgroup (e.g., *Desulfitobacterium dehalogenans* and *Desulfitobacterium frappieri*) (4, 8, 12, 20, 35). Although species related to the *Thermotogales* subgroup have not been previously implicated in reductive dechlorination, several members of this phylum are capable of S^0 reduction. Another species that was detected in *ortho*-dechlorinating enrichment cultures had the highest sequence similarity to the deeply branching species *Dehalococcoides ethenogenes*, which has been described as an obligate perchloroethylene-dechlorinating species (25). The consistent detection of this species in actively PCB-*ortho*-dechlorinating cultures and its absence from nondechlorinating cultures present the intriguing possibility that other obligate dehalogenating species exist.

In summary, SEMM has been shown to be an effective approach for developing community profiles associated with specific PCB-dechlorinating activities in a minimal defined medium. By using this approach, we have demonstrated that highly defined *ortho*-dechlorinating enrichment cultures have been developed and a stable microbial community has been maintained throughout sequential transfers in minimal growth conditions. Based on nutrient requirements of known species closely related to species identified in these *ortho*-dechlorinating enrichment cultures, efforts are currently under way to isolate and further characterize species from the enrichment community to confirm their role in catalysis of the dechlorination process.

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Microbial Dechlorination of 2,3,5,6-Tetrachlorobiphenyl under Anaerobic Conditions in the Absence of Soil or Sediment

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Bacterial enrichment cultures developed with Baltimore Harbor (BH) sediments were found to reductively dechlorinate 2,3,5,6-tetrachlorobiphenyl (2,3,5,6-CB) when incubated in a minimal estuarine medium containing short-chain fatty acids under anaerobic conditions with and without the addition of sediment. Primary enrichment cultures formed both *meta* and *ortho* dechlorination products from 2,3,5,6-CB. The lag time preceding dechlorination decreased from 30 to less than 20 days as the cultures were sequentially transferred into estuarine medium containing dried, sterile BH sediment. In addition, only *ortho* dechlorination was observed following transfer of the cultures. Sequential transfer into medium without added sediment also resulted in the development of a strict *ortho*-dechlorinating culture following a lag of more than 100 days. Upon further transfer into the minimal medium without sediment, the lag time decreased to less than 50 days. At this stage all cultures, regardless of the presence of sediment, would produce 2,3,5-CB and 3,5-CB from 2,3,5,6-CB. The strict *ortho*-dechlorinating activity in the sediment-free cultures has remained stable for more than 1 year through several transfers. These results reveal that the classical microbial enrichment technique using a minimal medium with a single polychlorinated biphenyl (PCB) congener selected for *ortho* dechlorination of 2,3,5,6-CB. Furthermore, this is the first report of sustained anaerobic PCB dechlorination in the complete absence of soil or sediment.

Anaerobic dechlorination of polychlorinated biphenyls (PCBs) has been demonstrated in situ and with laboratory microcosms containing sediment (reviewed in reference 1a). However, sustained PCB dechlorination has never been shown to occur in the absence of soil or sediments. Morris et al. (6) demonstrated a sediment requirement for the stimulation of PCB dechlorination within freshwater sediment slurries. Wu and Wiegel have recently described PCB-dechlorinating enrichments which required soil for the successful transfer of PCB-dechlorinating activity (9). In addition, no anaerobic microorganisms that dechlorinate PCBs have been isolated or characterized, and this may be due in part to the soil or sediment requirement. The inability to isolate dechlorinating organisms or maintain dechlorination without sediment has limited biogeochemical and physiological investigations into the mechanisms of PCB dechlorination.

Dechlorination (*ortho*, *meta*, and *para*) of single PCB congeners has been observed following anaerobic incubation of Baltimore Harbor (BH) sediment under estuarine or marine conditions (2). While sediments from several sites within BH are contaminated with PCBs (1, 5), background contamination of sediment is not necessarily a prerequisite for the development of PCB dechlorination in laboratory microcosms. Wu et al. (8) recently demonstrated *meta* and *ortho* dechlorination of Aroclor 1260 when it was added to the same BH sediments. These results showed that more than one dechlorinating activity could be developed with these sediments. It has been proposed that discrete microbial populations are responsible for specific PCB dechlorinations (1a). Consistent with this idea, the *ortho* dechlorination observed with BH sediments may be catalyzed by discrete microbial populations. In addition, these organisms may be able to couple PCB dechlorination with growth. Therefore we have attempted to select for *ortho* PCB-dechlorinating organisms by enrichment under minimal conditions with high levels of 2,3,5,6-tetrachlorobiphenyl. We also speculated that given the proper conditions, a PCB-dechlorinating population could be maintained in an actively dechlorinating state in the absence of sediment. Here we report that a distinct PCB-dechlorinating activity, namely, *ortho* dechlorination, was selected for through sequential transfer initiated with sediments from BH and sustained in the absence of soil or sediment. This is the first report of sustained anaerobic PCB-dechlorinating activity in the total absence of sediment.

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MATERIALS AND METHODS

Sediment samples. Sediment samples were collected with a petite Ponar grab sampler from a subsurface depth of 9.1 m in the northwest branch of BH (39°16.8′N, 76°36.1′W). An oily slick and gas bubbles formed at the surface immediately after the sampler disturbed the sediments. Sediments had a black coloration, a gelatinous texture, and a strong petroleum odor. The combined contents of the sampler were transferred to 0.95-liter canning jars (Ball Corporation, El Paso, Tex.). The jars were filled to the top and immediately sealed with dome tops and ring seals to exclude air. The samples were stored at ambient temperature in the dark prior to use.

Culture conditions. All sterile media in these experiments included an estuarine salts medium without sulfate (E-Cl) and were prepared anaerobically in an atmosphere that contained N_2-CO_2 (4:1) as previously described by Berkaw et al. (2). Briefly, the medium contained the following constituents, in grams per liter of demineralized water: Na_2CO_3, 3.0; Na_2HPO_4, 0.6; NH_4Cl, 0.5; cysteine-HCl · H_2O, 0.25; Na_2S · $9H_2O$, 0.25; $MgCl_2$ · $6H_2O$, 0.1; $CaCl_2$ · $6H_2O$, 0.1; and resazurin, 0.001. In addition, vitamin and trace element solutions (1% [vol/vol] each) were added (7). The final pH of the medium was 6.8. Media were dispensed into anaerobic culture tubes (18 by 160 mm; Bellco Glass, Inc., Vineland, N.J.) or 150-ml serum bottles (Wheaton, Millville, N.J.) sealed with Teflon-lined butyl stoppers (The West Co., Lionville, Pa.) that were secured with aluminum crimp seals (Wheaton).

Primary sediment enrichment cultures were generated in culture tubes by adding 2 ml of BH sediment to 8 ml of sterile E-Cl medium (approximately 5%, wt/vol [dry weight], sediment concentration), plus a mixture of sodium acetate, propionate, and butyrate to final concentrations of 2.5 mM each. Congener 2,3,5,6-tetrachlorobiphenyl (2,3,5,6-CB) was solubilized in acetone and added to each culture to a final concentration of 173 μM (50 ppm), and this resulted in a

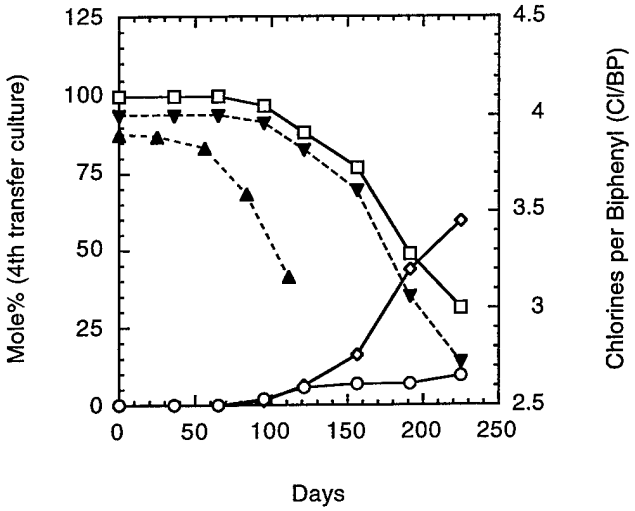

FIG. 3. Chlorines-per-biphenyl data for fourth- and fifth-sequential-transfer cultures without sediment. Mole percent data are given for the fourth-transfer culture. All data are given as the averages from duplicate cultures. Symbols: ◇, mole percent for 3,5-CB; ○, 2,3,5-CB; and □, 2,3,5,6-CB. ▼, chlorines per biphenyl of fourth-sequential-transfer culture; ▲, chlorines per biphenyl of fifth-sequential-transfer culture.

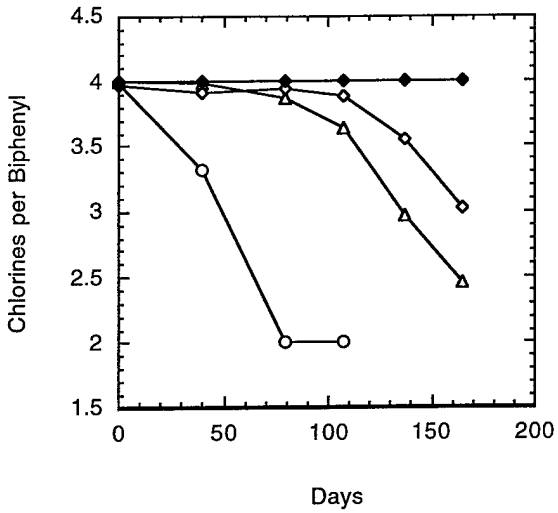

FIG. 5. Cultures with 2,3,5,6-CB and 1.0% (○), 0.1% (△), and 0.05% (◇) (wt/vol [dry weight]) sterilized BH sediment. Supernatant from a 5.0% sediment culture was sequentially transferred with 1.0, 0.1, and 0.05% (wt/vol [dry weight]) BH sediment in E-Cl medium, incubated for 4 months, and transferred again under identical conditions. The data presented represent the second set of transferred cultures. The chlorines-per-biphenyl data for the killed-cell control with 1.0% sterilized BH sediment are for a single culture (◆). The data from the live BH cultures are the average of duplicates.

these cultures into identical sediment-free media and still maintain dechlorinating activity.

The appearance of dechlorination at the fourth transfer of the sediment-free cultures after an incubation period exceeding that of earlier cultures in the transfer series suggests that the transfers were made too quickly (at low cell density) during the early part of the enrichment process. OD data for a later set of active sediment-free cultures (Fig. 4) revealed that significant dechlorination does not occur until the OD_{600} exceeds 0.2. This observation supports our conclusion that the ability to maintain good dechlorination earlier on in the sediment-free enrichment series was hindered by premature transfer of the cultures at low turbidity. Perhaps the earlier transfers at lower turbidity had prevented the development of hearty dechlorinating cultures and sustainability was simply an issue of low numbers of dechlorinators among the total population. The possibility that the organisms responsible for the dechlorination needed an extensive amount of time to adjust to the altered conditions (lack of sediment) before being able to carry out the dechlorination also exists. This latter possibility may be associated with the uptake (availability) of the PCB or supply of a nutrient. It is also possible that during this lengthy process we enriched for a prototroph that no longer requires a component of the sediment in order to dechlorinate a PCB.

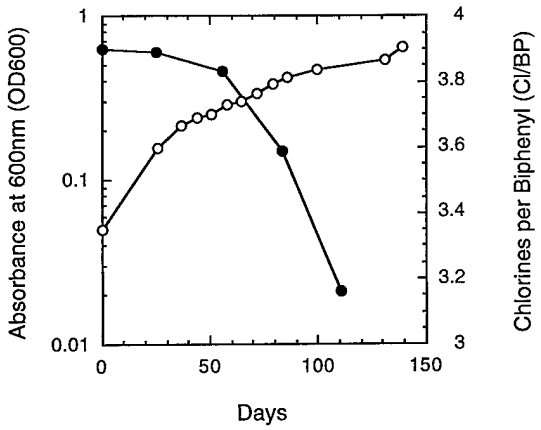

FIG. 4. OD (○) and chlorines-per-biphenyl (●) data from duplicate fifth-sequential-transfer cultures without sediment.

Sediment stimulation of *ortho* dechlorination. The above results demonstrate that *ortho* dechlorination is independent of the sediment. However, several results show the sediment to have a stimulatory effect. The first suggestion of this was observed with the decrease in the rate and extent of *ortho* dechlorination that accompanied the shift from *meta*-and-*ortho* to strictly *ortho* dechlorination (Fig. 1 and 2). This occurred after a primary culture had been transferred to a medium with far less sediment (5.0 to 0.1%, wt/vol [dry weight]). This change in activity could have been due to the decrease in the amount of sediment present. To examine this, a range of sediment concentrations was tested under the conditions described above. In order to be certain of the sediment concentration, the supernatant from the primary culture was transferred (10% [vol/vol]) into vessels containing E-Cl medium with the different amounts of BH sediment to be tested. After 4 months of incubation, transfers were made from these cultures into identical medium and the results of this second set of cultures are presented in Fig. 5. While dechlorinating activity could be maintained regardless of the sediment concentration, the lag preceding dechlorination increased to more than 100 days when the sediment concentration was lowered to 0.05% (dry wt). The cultures incubated with 1.0% (dry wt) sediment exhibited a higher rate of dechlorination and a shorter lag time than did those incubated with lesser amounts of sediment. Killed-cell controls (sterilized sediment cultures) exhibited no dechlorination. From a qualitative perspective, dechlorination did not change with sediment concentration and remained strictly *ortho*. Additional experiments with sediment-free cultures also demonstrated that the sediment could be stimulatory. Pre-dechlorination sediment-free cultures (in this case the

and three with mutations disrupting peptidoglycan biosynthesis, indicating the importance of cell envelope integrity in biofilm formation.

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Molecular Assessment of the Effect of PCBs on the Microbial Community Structure within an Enrichment Culture

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Polychlorinated biphenyls (PCBs) are ubiquitous environmental pollutants. However, the microorganisms responsible for anaerobic dechlorination of PCBs have not been identified. Understanding the microbial community structure would be useful for identifying an acceptable bioremediation approach. It has been hypothesized that a succession of different microorganisms is responsible for the dechlorination of complex mixtures of PCBs. If this is so, then the structure of a microbial community may be altered by each PCB congener that becomes available to the microorganisms. This was tested in a simple laboratory system with an enrichment culture that had been sequentially transferred with just one PCB congener added to the medium, 2,3,4,5-tetrachlorobiphenyl. Since this congener was routinely dechlorinated to several products, sequential transfers from the initial culture were made with each dechlorination product added to the medium. After several transfers under these conditions and after dechlorinating activity had been well established, the population profiles were examined by denaturing gradient gel electrophoresis (DGGE) and by amplified ribosomal DNA restriction analysis (ARDRA). The results of these examinations indicate that the addition of a PCB causes both a reduction in the microbial diversity and a shift in the population profile. DGGE and ARDRA analyses also show that the shifts in population are distinct for individual congeners, with a reduction in microbial diversity observed when fewer dechlorination reactions occur. Overall, the results indicate that PCBs could have a significant effect on the microbial ecology of an impacted site.

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Evidence that the bacterial symbiont "*Candidatus* Endobugula sertula" plays a role in bryostatin biosynthesis in the marine bryozoan *Bugula neritina*.

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Bugula neritina is a marine bryozoan that harbors an uncultivated symbiont, the gamma proteobacterium "*Candidatus* Endobugula sertula." *B. neritina* is the source of the bryostatins, a family of macrocylic lactones with anti-cancer activity. Bryostatins are complex polyketides similar to bacterial secondary metabolites biosynthesized by modular Type I polyketide synthases (PKS-I). We are investigating the possibility that "*E. sertula*" is the biosynthetic source of bryostatins in *B. neritina*. We obtained a bacterial PKS-I gene fragment (KSa) that is present in all *B. neritina*, and specific to *B. neritina*, suggesting that it could be part of the bryostatin synthesis pathway. Bryostatin activity, "*E. sertula*" levels and KSa signal were measured in different portions of *B. neritina* colonies and there was a correlation between KSa signal and "*E. sertula*" levels. Settling larvae were treated with antibiotics to greatly reduce "*E. sertula*" levels and then allowed to grow in non-sterile seawater for 3 months to re-establish populations of commensal bacteria. Denaturing gradient gel electrophoresis of treated and untreated control *B. neritina* colonies showed that "*E. sertula*" was reduced in treated colonies and other bacteria were not. Treated *B. neritina* grew as fast as untreated controls, but had significantly reduced levels of bryostatin activity and KSa signal. Based on the presence of a bacterial PKS-I in *B. neritina*, and the link between bryostatin activity, "*E. sertula*" and KSa, we propose that "*E. sertula*" is an excellent candidate for a microbial source of bryostatins in *B. neritina*.

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Response of an Indigenous Soil Microbial Community Following Contamination with the Explosive RDX

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A clean loam soil was artificially contaminated with the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) to observe the changes in the microbial community structure upon contamination. Initial soil microcosm experiments demonstrated an initial lag period of 25 days followed by 80% mineralization of RDX after 125 days. The observed lag period suggested that changes in the microbial community structure were required for RDX mineralization to occur. Denaturing gradient gel electrophoresis (DGGE) was performed on 16S rDNA fragments amplified from total community DNA, and patterns between the control and contaminated columns were compared. Subtle differences in banding patterns were observed and 5 bands that were present in the contaminated soil but not in the clean control were